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Studies on the Activities of Infective Hookworm Larvae
with Special Reference to Vertical
Migration

Dissertation

Submitted to the Director of the School of Hygiene and
Public Health of the Johns Hopkins University in Con-
formity with the requirements of the degree of Doctor
of Science in Hygiene.

Florence King Payne

June 1923

Introduction.

These studies were undertaken for the purpose of determining the limits of vertical migration of hookworm larvae under various conditions and the object was to obtain information which could be applied to the solution of problems in hookworm control. Preliminary experiments were carried out in Trinidad from May to December, 1921. These experiments were extended and studies under field conditions were added in Porto Rico during the summer of 1922. The results of these two series of experiments are incorporated in parts I and II of this paper.

The results of the preliminary series of experiments showed the need of a more accurate knowledge of the physical and biological factors involved in migration, particularly with regard to the stimuli which initiate the activity and the mechanism by which it is accomplished. The third section of this paper deals with these points. In the preliminary experiments it was determined that active migration very rapidly reduced the stored nutritive material in the larvae. The experiments reported in the fourth part of the paper were undertaken to determine the effect of this reduction in the supply of nutritive

material on the power of the larvae to establish themselves in the host.

The parts of the paper are as follows:

Part I. Vertical Migration of Infective
Hookworm Larvae in the Soil.

Part II. Field Experiments on Vertical
Migration of Hookworm Larvae.

Part III. Studies on Factors involved
in Migration of Hookworm Larvae
in Soil.

Part IV. The Relationship of Physiological
Age of Hookworm Larvae to their
Ability to Infect the Human Host.

The author wishes to make grateful acknowledgment to Dr. W. W. Cort, for assistance and counsel in the prosecution of these studies. Much help was given by all members of the Trinidad and the Porto Rico expeditions. Special acknowledgment is due to Mr. N. R. Stoll and to Dr. N. A. Cobb for assistance which is described in the text. Dr. Joseph S. Ames and Dr. William H. Howell gave helpful advice in the interpretation of observations with physical and physiological relationships.

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I. VERTICAL MIGRATION OF INFECTIVE HOOKWORM LARVAE IN THE SOIL IN TRINIDAD, B. W. I.

Introduction.

There have been a number of studies of the migration of hookworm larvae on the surface of soil and on the walls of mine shafts, but investigations of their travels beneath the surface have been hampered by the lack of adequate means of isolating them from surrounding media. The development of a successful method of isolation (Cort, Augustine, Ackert and Payne, 1922; Baermann, 1917) has given an opportunity for the pursuit of studies of such migrations.

The vertical migrations of hookworm larvae may affect practical hookworm control in two chief ways: First, by the possible retirement of the larvae during dry weather from the surface to zones of more moisture. Second, by the possible migration upward of larvae which are beneath the surface. Studies of such migrations should assist in the evaluation of methods of excrement disposal, especially where burial is practiced or pit latrines are used. The present study considered only the second phase of vertical migration. Each experiment was based on the assumption that excrement containing

fully developed larvae had been buried at a known distance beneath the surface. The object was to determine whether larvae were likely to reach the surface in sufficient numbers to produce an infective focus.

Methods.

Samples of the three types of soil which were used were obtained in quantities of fifty pounds to one hundred pounds. Each was thoroughly mixed to obtain uniform distribution of the various constituents. Portions of each sample were dried at a temperature of about 60° C. and were pulverized. It was determined by experiment that drying at this temperature was sufficient to kill any nematodes or larvae, but was not high enough to char organic matter. The dried soil was kept as the stock from which material for the various experiments was obtained.

Containers for the experiments were of various sizes. The ones most used were tin sleeves, about three inches in diameter and five inches high. They were slightly tapered so that two or more could be joined to obtain additional length. Other containers of various sizes and forms were used in a few experiments and will be described with them.

Except where otherwise specified, only well developed Necator americanus larvae were used. The methods of culture and of isolation of the larvae have been given in an earlier paper of this series (Cort, et al., 1922). In nearly all experiments the larvae were counted and were placed in a thin layer of moist soil on the bottom of the container.

The determination of the extent of upward migration accomplished by the larvae was made by removing the soil from the container in layers of measured thickness. Each layer was placed in an isolation apparatus and the larvae were recovered from it according to the method to which reference has been made. The larvae recovered from each layer were counted.

Experimental Work.

Experiments were conducted to test the effect of the following factors on the upward migration of hookworm larvae:

1. Moisture content.
2. Conditions simulating the rise of
ground water.
3. Type of soil.

Another series of experiments was made to study the disappearance of nutritive material from the bodies of the larvae at various stages of migration.

The first experiments were designed to test the effect on vertical migration of different degrees of moisture, uniformly distributed through the soil. In all of these experiments soil from the same lot was used. It was typical of the soil in the region of Princes' Town and was made up of a mixture of marl, clay, and humus. For convenience it is designated in the description of the present experiments as "garden soil." The standard tin sleeve containers were used.

The experiments were performed during the rainy season and specimens of soil obtained from fields which were known to harbor hookworm larvae contained from 33.5 to 48.7 per cent. of water. In the laboratory, however, it was found impracticable to prepare a mixture of dried soil and water with uniform distribution, in which there was less than 40 per cent. of water. In the experiments with waterlogged soil the earth was saturated throughout, and additional water was added so that the surface of the sample was always submerged.

Table 1 shows the results of a series of ten experiments which were made to test the influence of the moisture content on vertical migration of hookworm larvae. From specimens of which the moisture content was 40 to 42.7 per cent. (Table 1, Experiments 1-7), 1877 larvae were recovered. Of these, 1798 or 95.7 per cent. were in the lowest inch of the specimen; that is,

TABLE 1.

Influence of moisture content on vertical migration of hookworm larvae.
 (The zones are measured from the bottom of the dirt in the containers, and represent the distances above the place of introduction of the larvae.)

Experiment.	Moisture in per cent.	Days.	Larvae incubated.	Zone 1.	Zone 2.	Zone 3.	Larvae recovered from Zone 3.	Zone 4.	Larvae recovered from Zone 4.	Zone 5.	Larvae recovered from Zone 5.	Zone 6.	Larvae recovered from Zone 6.	Zone 7.	Larvae recovered from Zone 7.
1	41.4	4	507	0-1"	51 3'-1"	147 1"-1"	9 1'-1"	13'-2½"	4	2½"-8"	0	3½"-4"	0	0	0
2	41.4	9	611	0-1"	30 3'-1"	7 1"-1"	2 1'-1"	13'-2½"	2	2½"-3½"	0	3½"-4"	0	0	0
3	40.0	6	455	0-1"	14 3'-1"	0 1"-1"	0 1'-1"	11'-2"	0	2"-2½"	0	2½"-3½"	0	3"-4"	0
4	42.2	4	4400	0-1"	962 3'-1"	83 1"-1"	25 1'-1"	11'-2"	9	2"-2½"	0	2½"-3½"	0	0	0
5	40.6	13	995	0-1"	0 3'-1"	0 1"-1"	0 1'-1"	11'-2"	0	2"-2½"	0	2½"-3½"	0	0	0
6	42.7	7	4530	0-1"	345 3'-1"	51 1"-1"	1 1'-1"	11'-2"	12	2"-2½"	3	2½"-3½"	4	3"-3½"	2
7	40.6	6	560	0-1"	91 3'-1"	17 1"-1"	0 1'-1"	11'-2"	0	2"-2½"	6	2½"-3½"	0	0	0
8	Water-	7	1137	0-1"	2 3'-1"	0 1"-1"	0 1'-1"	11'-2"	0	2"-2½"	0	2½"-3½"	0	0	0
9	logged	3	347	0-1"	2 3'-1"	2 1"-1"	1 1'-1"	2½"-3½"	0	3½"-4½"	0	3½"-4½"	0	0	0
10		2	1500	0-1"	163 3'-1"	5 1½"-2½"	0 1'-1"	2½"-3½"	0	3"-3½"	0	3"-3½"	0	0	0

in the periods of four to thirteen days between inoculation and recovery there was no evidence of effective migration. From the specimen left for nine days, only 6 per cent. of the larvae originally used were recovered, and from the specimen left for thirteen days no larvae were recovered.

From specimens which were water-logged (Table 1, Experiments 8-10), 175 larvae were recovered. Of these, only 8 had migrated more than three fourths of an inch. The supernatant water on these specimens was examined daily for larvae and none was found in any experiment. It would appear that the death rate in these specimens was high.

The failure of extensive migration to occur under conditions of relatively uniform distribution of moisture led to the attempt to simulate some conditions existing in nature. Various efforts were made to bring about experimental conditions which would resemble those existing in the soil during the rise of ground water from the water table to the drying surface.

In experiment No. 11 (see Table 2), dry soil was placed in a glass tube three eighths of an inch in diameter to a depth of three and one-half inches. More than one thousand larvae were placed in the lower end on a little moist earth. This end was closed with a stopper into which was inserted a

TABLE 2.
Influence of rise of ground water on vertical migration of hook-worm larvae.

Experiment	Days	Larvae hatched	Zone 1	Larvae received from Zone 1	Zone 2	Larvae recovered from Zone 2	Zone 3	Larvae recovered from Zone 3	Zone 4	Larvae recovered from Zone 4	Zone 5	Larvae recovered from Zone 5	Zone 6	Larvae recovered from Zone 6	Zone 7	Larvae recovered from Zone 7	Zone 8	Larvae received from Zone 8
11	1	1000	0-1"	0-1"	0-1"	100	1-1"	36	1-2"	39	2 1/2"-3 1/4"	8	3 1/2"-5 1/4"	345				
12	8	600	0-1"	0-1"	1-2"	1	2-3"	12	3-3 1/4"	53	3 1/4"-4"	172	4"-4 1/2"	32				
13	4	?	0-1"	0-1"	2-3"	2	3-3"	5	3-4"	6	4"-5"	37	5"-5 1/2"	105	5 1/2"-6 1/4"	46		
14	10	1530	0-1"	0-1"	1 1/2"-2 1/4"	1	2 1/2"-3 1/4"	1	3 1/4"-4 1/2"	3	4 1/2"-5 1/4"	10	5 1/2"-6 1/4"	11	6 1/2"-7"	1	7 1/2"-8"	

very slender glass tube bent in the form of a U. The tube of earth was then placed in a vertical position. Water was introduced, a few drops at a time, through the U tube. Care was taken that the level of the water in the U tube should not be higher than the lower end of the column of earth in the larger tube. In the course of a few hours water had permeated throughout the soil. Of 345 larvae which were found in the top one fourth of an inch of the earth on the following day, 117 were recovered from the uppermost lump of earth which measured about one eighth of an inch in diameter.

In experiment No. 12 (see Table 2), one of the standard tin sleeves was used. This was set on a flat tin surface and the larvae were placed in the bottom in a bit of moist earth. Dry earth was then poured into the sleeve to a depth of four inches, and in the center additional earth was piled to form a cone one half inch in height. A little water was placed on the flat surface outside the container, and it was renewed as rapidly as it was taken up by the soil. The surface of the soil in this container was examined from day to day. After twenty-four hours it was estimated that about 300 larvae could be found with a binocular microscope. The container was kept open and no attempt was made to prevent drying of the surface. After a few days only empty sheaths were visible on the surface of the higher lumps of earth, while larvae could be seen at

lower levels. A control experiment was conducted in the same manner, but without larvae, and on the eighth day the moisture content of the soil was found to be 42.3 per cent.

In order to eliminate the possibility of larvae ascending the walls of the container instead of migrating through the earth, experiment No. 13 (see Table 2) was performed, with a container five inches square. This experiment was carried out in the same manner as No. 12, but drying for such a long time was not permitted.

In experiment No. 14 (see Table 2) there was used a deeper layer of earth than in the previous experiments, and in order to obtain a continuous water column from the bottom to the top, it was necessary to moisten the top layers of earth.

In the four experiments recorded in Table 2 migration was more successful. Of 1094 larvae recovered, only 165 or 15.1 per cent. were found within one inch of the bottom of the container. 814 or 74.4 per cent. had migrated three inches or more before recovery; 235 had migrated four inches or more, and 169 five inches or more. The greatest distance noted was in the case of three larvae, in experiment No. 14, which were recovered from zone seven and one half to eight and one half inches from the bottom.

The experiments which have been described involved studies of migration in only one type of soil, and this a type

which is common in relatively restricted areas. In order to determine whether migration might be more successful in other soils a preliminary series of experiments was made with two others which were available at the time. One of these was composed almost entirely of very fine white sand, the other was of sand mixed with finely divided particles of red clay. The experiments with soils were conducted in small containers with a depth of soil of about two inches. The technique was identical with that described in the experiments above which simulated the rise of ground water, except that the larvae were not recovered. The success of migration was tested by frequent search for larvae on the surface of the soil by means of a hand lens or binocular. Each experiment was controlled by a similar preparation in which garden soil was used.

Six series of experiments were made in the attempt to establish conditions under which successful migration would occur in these soils. The experiments with the white sand were uniformly unsuccessful. No larvae were found on the surface of this soil. In the case of the mixture of sand and red clay, there were found at the end of twenty-four hours in each experiment a few larvae, but never so many as were seen on the garden soil control.

The results of the experiments which have been described indicate that in the types of soil which have been studied

vertical migration takes place through only a limited range, except under the most favorable conditions. Studies of the larvae, used in the experiments, showed that there was little or no migration, if the food granules were sparse, and anatomical details were readily made out. Larvae, which in the beginning were so thickly studded with granules that very little internal structure could be distinguished, showed, after migration of a few inches a great diminution in granules and a general appearance similar to that pictured by Looss (1911) in a larva reared in a medium with insufficient nutriment. These observations led to a series of experiments to study the diminution in the number of granules in relation to the various distances migrated.

Six different lots of well developed and nourished larvae were used for this experiment, and with each lot several preparations of different depths were made according to the technique described for the experiments simulating the rise of ground water. The distribution of food granules was studied in the larvae of each lot, in the larvae which came to the surface of the preparations, and in the larvae which were recovered from various zones of the deeper preparations. Photomicrographs were made of typical larvae of each lot and of larvae from each stage of migration. The preparation of the photomicrographs was done by Dr. Geo. C. Payne, and

acknowledgment is made to him for his assistance as well as to Dr. K. S. Wise, Surgeon-General, and Dr. Baeza, Acting Government Bacteriologist, for the use of necessary apparatus.

The experiments were conducted under fairly uniform temperature conditions. Room temperature varied from about 72 to 87° F. All experiments were carried through in as short a time as was consistent with the height of migration which was desired. Even the highest migration took place within less than a week from the preparation of the lot, and four days from the actual inoculation of the soil. Portions of the supply of stock larvae were retained in each instance and were kept in clean water as controls to show the changes in the distribution of food granules in inactive larvae. Observations on the controls showed that there was no appreciable diminution in the number of food granules during the period of any experiment. In interpreting these experiments it is to be understood that the total distance travelled by a larva is much greater than the vertical distance of apparent migration. Hand lens studies of the activities of larvae in vertical glass tubes containing earth showed that a larva frequently traverses the same zone many times before finally leaving it for a higher one.

Diminution in the number of food granules was shown after migration of one inch, which was the smallest distance at which studies were made, and when only eighteen hours had elapsed

from the time of inoculation. The loss of granules was apparently progressive and constant with farther migration and with the passage of more time, so long as activity was maintained. Because of the devious courses taken by the larvae in their travels there was a certain amount of variation in the diminution of granules in larvae from the same lot and the same zone, but when representative individuals were selected from each zone the disappearance of the granules could be followed with a fair degree of accuracy. There appeared to be an early diminution in the number of granules in the anterior portion of the larvae, and this was followed by an apparent increase in the width of the lumen of the intestine, probably due to a decrease in depth of the intestinal cells. The accompanying figures show a characteristic series of such changes in larvae from one lot, and from different zones of migration up to six and three fourths inches (See Plates XI to XIII, Figs. 1-11).

SUMMARY.

1. In garden soil with uniform conditions of moisture throughout the sample very few N. americanus larvae migrated more than two inches above the point of inoculation.
2. In water-logged garden soil there was apparently a very high death rate and there was little migration.
3. In garden soil in which the conditions of rising ground water were simulated 74.4 per cent. of the larvae recovered

had migrated three inches or more, 15.4 per cent. had migrated five inches or more while a few had migrated more than seven and one half inches.

4. Migration was apparently more difficult in the specimens of fine sand and the mixture of clay and sand than in the specimens of garden soil.

5. The food supply carried by N. americanus larvae apparently restricts the range of their vertical migration to narrow limits, since a distinct reduction in the granules in the intestinal wall could be noted after vertical migrations of only an inch or two, and almost a complete loss of these granules, came about in those which had migrated for five or six inches.

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PLATE XI.



FIG. 1.



FIG. 2.



FIG. 3.



FIG. 4.

PLATE XII.



FIG. 5.



FIG. 6.

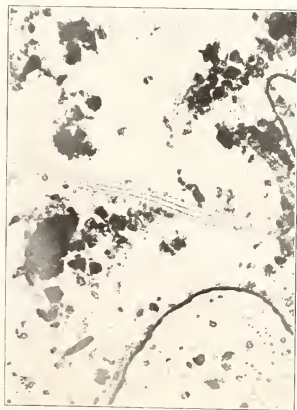


FIG. 7.

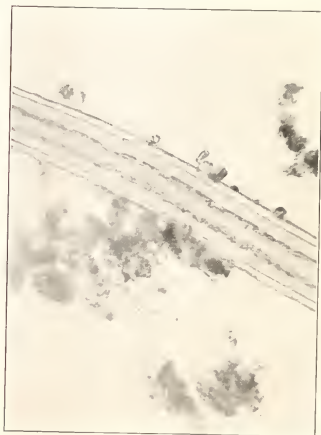


FIG. 8.

PLATE XIII.



FIG. 9.



FIG. 10.

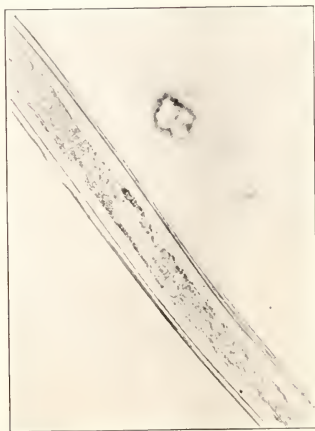


FIG. 11.

EXPLANATION OF PLATES.

Plate XI.

- Fig. 1. Larva from lot No. 4 before migration, low-power.
- Fig. 2. Larva from lot No. 4, before migration, high-power.
- Fig. 3. Larva from lot No. 4, after migrating a vertical distance of two inches, low-power.
- Fig. 4. Larva from lot No. 4, after migrating a vertical distance of two inches, high-power.

Plate XII.

- Fig. 5. Larva from lot No. 4, after migrating a vertical distance of three and one half inches, low-power.
- Fig. 6. Larva from lot No. 4, after migrating a vertical distance of three and one half inches, high-power.
- Fig. 7. Larva from lot No. 4, after migrating a vertical distance of four and one half inches, low-power.
- Fig. 8. Larva from lot No. 4, after migrating a vertical distance of four and one half inches, high-power.

Plate XIII.

- Fig. 9. Larva from lot No. 4, after migrating a vertical distance of six and one half inches, low-power.
- Fig. 10. Larva from lot No. 4, after migrating a vertical distance of six and three quarters inches, low-power.
- Fig. 11. Larva from lot No. 4, after migrating a vertical distance of six and three quarters inches, high-power.

II. FIELD EXPERIMENTS ON VERTICAL MIGRATION OF HOOKWORM LARVAE IN PORTO RICO.

Introduction.

The efficiency of certain methods of hookworm control depends on the inability of hookworm larvae developing at the point of disposal of the excrement to migrate to locations whence they may be transferred to man. This is notably true of methods which involve the burial or trenching of excrement collected in latrines of the pail type. This system is in use in many countries within the zone where hookworm disease is prevalent. It has seldom been questioned that careful burial of infective night soil would effectively eliminate all sources of danger. Dershimer, however, reported finding larvae on the surface of soil in which feces were said to have been buried at a depth of two feet or more (Annual Report, International Health Board, 1919, p. 118). Ashford and Gutierrez Igaravidez (1911, p. 112) noticed a very severe infestation in a negro who was employed to bury the nightsoil from an anemia hospital. The patient himself attributed his infection to that "hole in the ground."

In 1921 an investigation was begun in Trinidad, British West Indies, to determine from what depth infective hookworm

larvae would migrate to the surface (Payne, 1922). In this investigation three types of soil were used. One, a loam in which there was much clay, the other two, mixtures of clay and fine sand. The experiments were all conducted in the laboratory and only mature hookworm larvae were used. It was noted in these investigations that migration proceeded more readily when there was definite upward movement of the water column. Under the most favorable conditions attained in these experiments the highest migration accomplished was eight and one half inches above the level of burial.

During the summer of 1922 a series of laboratory experiments like those conducted in Trinidad was begun in Utuado, Porto Rico, using a sandy loam soil of a type which is very common in Porto Rico. Mature larvae of *N. americanus* were used. For these experiments there were used glass tubes about two inches in diameter and four inches in length, in which the progress of moisture and of migrating larvae could be observed. For the longer distances of migration the tubes were joined in vertical columns of the desired length. The larvae were placed on a layer of soil two inches deep in the bottom of the column and dry soil was poured over them to the required depth and was lightly tamped. The soil was then slowly moistened. It was

observed that the larvae migrated through this soil with ease. In one case larvae were buried at a depth of six inches on July 21 at 6:30 and on July 22 at 7:30 A. M. or thirteen hours after burial larvae were numerous on the surface. Larvae buried at a depth of ten inches on July 21 appeared on the surface July 23. These observations indicated that it was imperative that investigations should be carried out on a scale and under conditions that would simulate actual practice in the disposal of excrement by burial. Therefore a series of field experiments was planned in which vertical migration could be tested in tubes of large size and with large numbers of larvae.

Methods.

In the field experiments every effort was made to test conditions which would be met in actual practice in the disposal of human excrement by burial. Two plots of ground were selected. The first was in a garden and was exposed to the sun throughout the day. All vegetation was cleared from this plot. The second was in a field in which a forage grass was cultivated. This grass was very thick and reached a height of seven feet or more. As a result the soil between the rows of grass was always in deep shade. The vegetation between the rows was cleared before starting the experiments. The soil of the two plots selected were apparently identical. The type was an

easily worked sandy loam containing some humus, but apparently very little clay. No attempt was made to vary the natural supply of moisture. There were frequent showers which usually came in the afternoon.

A few tin tubes about ten inches square were used for shallow burials, and for the remainder of the burials galvanized iron tubes twelve inches in diameter were utilized. The length of the tubes was such that they protruded from four to eight inches above the surface in order that no particles of earth might be carried out of the container by the splashing of heavy rains. In order to avoid the wash of surface water the hole for each burial was dug to accurately fit the tube which was to line it. There was no effort to pulverize the soil to a greater extent than would be the case in actual practice nor was the soil tamped after being placed in the hole. The nature of this soil, however, was such that the necessary handling broke up all large aggregates and left few lumps more than one inch in diameter. Experiments were also done in the exposed plot, in which the tubes were filled with clay instead of the soil which had been removed from the hole. Two types of clay were used, one locally known as white clay, was of a light gray color, composed of very fine particles with apparently no sand; the

second, a red clay in which there was a considerable amount of sand. After the burial was complete the experiments were protected from fowls and larger animals by wire netting covers over the protruding containers.

Before beginning the experiments samples of the soil from each of the selected plots were placed in the isolation apparatus (Cort, et al., 1922) and examinations were made for hookworm larvae. All of these samples yielded negative results. Examinations of similar samples were made at various times during the course of the experiments and those were likewise negative.

The material buried was of three classes,

- (a) Estimated numbers of well-nourished and active mature larvae.
- (b) Cultures containing mature larvae, used as checks on the migration of material (a)
- (c) Feces known to contain abundant ova of N. americanus.

The number of larvae isolated from cultures was estimated by suspending the total number in a measured quantity of water, between five and ten mils, depending upon the abundance of larvae, mixing thoroughly and withdrawing a measured quantity of the suspension, from 0.15 to 0.5 mil. This sample was placed on a microscopic slide and the larvae were carefully counted under a 16 mm. objective with a 10 ocular. From this result the total number

of larvae was estimated.

The number of hookworm ova in the specimens of feces which were buried was estimated in nearly all cases by a method devised by Mr. N. R. Stoll and described in another paper of this series (Stoll, 1923). The writer wishes to acknowledge indebtedness to Mr. Stoll for assistance in making these determinations and for supplying the data on which some of the estimates are based. In order to be assured that mature hookworm larvae could be produced from the ova in the specimens used, a small portion was removed from each specimen and was mixed with a little earth on the surface of the experimental plot. Each of these control cultures was protected by tin tubes which extended three inches below the surface and four inches above the surface and was covered with wire netting.

From the time of burial the surface of the soil within each tube was daily inspected with a hand lens for the appearance of larvae. As soon as larvae appeared in numbers the surface of the soil was carefully scraped off with a spoon, the material obtained was placed in an isolation apparatus and the number of larvae was estimated by the method mentioned. In a few cases the appearance of larvae, as determined with the hand lens, was delayed and specimens

of soil from the surface were examined by means of the isolation apparatus before larvae had been seen. Examinations of the surface were repeated at intervals of a few days until the numbers obtained became very small or the results were negative. In order to determine the distribution of the larvae at the close of the experiment a soil sampling tube, with a diameter of about two inches, was driven to the bottom of all the pits with a depth of ten inches or more. The soil withdrawn was divided into three portions designated as top, middle, and bottom, and the larvae in these portions were isolated and counted separately. The burials in clay were all relatively shallow and were also examined with a soil sampling tube, the sample being divided into two portions, top and bottom.

Results.

The daily inspection of the surface of the experiments yielded much information in regard to the time of appearance, distribution over the surface, and the activities of the larvae which were able to migrate the entire distance. Reference to Tables I, II, and III will show that there were only three cases in which larvae were isolated from the surface, but had not been previously seen with the hand lens. These studies

showed that the larvae were distributed over the entire surface of the soil in the tube. The only tendency to concentration was upon small lumps of earth which protruded above the general level (Augustine, 1922). On the edges of such particles the concentration was frequently such that the presence of larvae was readily ascertained with the unaided eye. It was observed in these studies that the majority of the larvae usually arrived at the surface at about the same time. That is, a few days after the first appearance of the larvae many new ones came up daily and then the numbers rapidly diminished. This observation was confirmed by quantitative studies in the isolation of the larvae.

Table 1 shows that mature hookworm larvae, buried in the sandy loam soil which was used in these experiments, were able to reach the surface from as great a depth as 24 inches. The time required to reach the surface increased with the deeper burial. The maximum speed noted was in the case of larvae buried at a depth of 10 inches in the exposed plot. Some of these larvae reached the surface two days after the burial, a vertical migration of five inches a day. The percentage of the larvae recovered from the surface decreased progressively with the increasing depth of burial. From larvae which were buried at a depth

TABLE I.
Vertical migration of mature larvae of N. americanus, buried in sandy loam soil.

Depth.	Exposure.	Estimated number of larvae buried.	Number of days until appearance of larvae.	Number of days until last isolation from surface.	Total number of larvae isolated from surface.	Percentage of larvae recovered.	Number of days until experiment.	Distribution of larvae at close of experiment.			Total recovered.
								Top zone.	Middle zone.	Bottom zone.	
4"	Sun	9,400	2	12	5,827	62					
6"	"	40,000	2	19	20,515	50					
8"	"	25,000	2	19	12,386	49	21	324	0	108	432
10"	"	40,000	2	19	13,471	35	31	720	0	0	720
10"	Shade	17,100	4	26	10,098	59	22	72	108	36	216
14"	Sun	15,360	5	18	2,136	14	29	36	0	0	36
14"	Shade	12,388	4	26	1,005	8.1	25	684	360	5,088	7,322
18"	Sun	36,500	5	20	1,314	3.6	24	0	12,960	0	12,960
18"	"	150,000	11	19	4	0.003	20	14,040	14,400	15,120	43,560
24"	Shade	—		20	15,520						

of 4 inches 62 per cent. were recovered, while from those buried at a depth of 18 inches 3.6 per cent. were recovered. The number which migrated from the 24-inch burial in the sun was very small, and, beside the additional depth, there probably were two factors which prevented effective upward migration in this experiment. The larvae used were obtained from an older culture than those used in the other experiments. These larvae had remained in the original culture for seventeen days, and probably were not in the best physical condition. Furthermore, a shower intervened between the digging of the hole for this burial and the covering of the larvae, so that the earth was wet. The fact that mature larvae can migrate in large numbers from a depth of 24 inches is shown by the results obtained from the burial of a culture containing mature larvae and the recovery of 15,520 larvae from the surface. This, however, does not represent the maximum capability of the hookworm larvae for vertical migration, as is shown in other experiments, the results of which are given in Table II, which show migration in numbers through a distance of 36 inches.

The results of the burial of feces containing hookworm ova, as shown in Table II, demonstrate that the conditions were most favorable for the development of larvae. After migration

TABLE II.
Development and migration of larvae of N. americanus from feces buried in sandy loam soil.

[illegible]

to the surface from depths of 4 inches to 8 inches there were isolated larvae which accounted for 22 to 28 per cent. of the ova buried. These results are better than those shown by the cultures on the surface which were planned as controls. The number of larvae yielded by these cultures were from 0.2 to 20.5 per cent. of the numbers of ova cultured. Even at a depth of 36 inches development proceeded readily. The time required for the appearance of larvae on the surface of the soil increased with the depths of burial, but the trend was not so constant as that in Table 1. This is probably due to the fact that not all larvae come to maturity in the same number of days, as well as to differences in the vigor of the larvae. The relationship of the number of larvae isolated to the number of ova buried showed a constantly decreasing percentage except in the case of the 36 inch burial in the shade which showed extraordinary development and migration. The number of larvae removed with the soil sampling tube on the eighteenth day from the beginning of this experiment indicates that, if the experiment had been allowed to run a few days longer, many more larvae would have appeared on the surface.

In the experiment in which counted hookworm larvae were buried under white clay, no larvae were recovered from the

surface. In order to check these findings a culture containing mature larvae was also buried and the results obtained from this experiment confirmed the inability of the larvae to reach the surface. In the two experiments with buried feces containing hookworm ova no larvae were isolated from the surface. At the close of the experiment the examinations to determine the distribution of the larvae showed, however, that there had been development of the larvae and that there was an attempt to migrate upward in the one, but not in the other experiment. The surface control culture for the negative case showed development of 0.2 per cent. of the ova cultured. In the red clay, which contained some sand, development and subsequent migration to the surface was successful. Of the ova buried 6 inches 2.9 per cent. developed and migrated to the surface, while in the burial of 10 inches 0.13 per cent reached the surface. While migration took place more readily in this clay than in the white clay, the results were still far below those obtained in the sandy loam at the same depth. It was also observed in the experiments carried out in Trinidad (Payne, 1922) that the success of migration varied with the type of soil used.

The distribution of the larvae as determined with

TABLE III.
Development and migration of larvae of N. americanus buried in clay soil.

Depth.	Type of clay.	Estimated number of larvae or ova buried.	Number of days until appearance of larvae.	Number of days until isolated from surface.	Total number of larvae isolated from surface.	Percentage of larvae isolated.	Number of days until experiment.	Distribution of larvae at close of experiment.		
								Top zone.	Bottom zone.	Total isolated.
6"	White	13,255 1	Not seen	16	0	0	26	216	540	756
6"	"	— 1	"	16	0	0	26	1,260	792	2,052
6"	"	Ova	"	16	0	0	26	0	0	0
6"	"	2,432,940	"	23	0	0	24	360	72	432
6"	"	551,500	"	21	12,990	2.9	22	2,160	1,080	3,240
6"	Red	447,756	10	11	23,100	0.13	12	78,840	23,760	102,600
10"	"	1,711,200	10							

the soil sampling tube at the close of the experiments (Tables I, II and III) indicates that even in the difficult white clay there was a definite effort on the part of the larvae to migrate upward. There is no doubt that if some of the experiments had been allowed to run for a longer time, many more larvae would have migrated to the surface. What the limiting factors were cannot be determined from the data now at hand, but it would appear that the structure of the soil had an important influence on the distance migrated. It is possible that a large number of these larvae wasted much of their food supply in fruitless wandering (Payne, 1922). In most cases the larvae isolated at the close of the experiment showed little granulation. They were transparent, inactive and appeared to be exhausted.

Experiments to Test the Possibility of Migration from Pit Latrines.

A preliminary experiment was performed to test the possibility of migration of hookworm larvae to the surface under conditions simulating a pit latrine. A circular hole about eighteen inches in diameter at the top, fourteen inches at the bottom, and about eighteen inches deep was dug. Feces containing abundant ova of N. americanus were smeared around

the bottom of the wall forming a band about two inches wide, the bottom being left clean. A galvanized tub was inverted over the hole, and a drainage ditch was dug in such manner as to remove all surface water. The condition of the feces on the wall of the pit was inspected daily. From the time that larvae might be expected to have attained maturity specimens were taken for examination in the usual manner with the isolation apparatus. The specimens consisted of scrapings from the surface of the ground around the pit and bands of soil from the wall of the pit. The examination of ants which soon became active in the feces showed that they carried away particles of the material. At one point on the wall of the pit they had constructed a run which extended from the bottom of the pit to the surface. Earthworm casts also appeared on the bottom and wall of the pit. The activities of the ants and earthworms were so effective that within a week it was impossible to distinguish the feces or to determine by inspection where they had been placed.

Examinations were made as follows :

Larvae isolated

August 13 (8 days from the beginning of the
experiment,) specimens from :

The ant run..... 1

A band, about two inches wide and three inches

above band of feces..... 622

A band, about two inches wide and one inch above the
band of the feces..... 7,600

August 17 (12 days from the beginning of the experiment), specimens
from :

Surface of ground around the top of the pit..... 0

A band, about three inches wide, thirteen inches above
band of feces 0

August 21 (16 days from beginning of experiment), specimens from:

A top band three inches wide..... 0

A middle band three inches wide..... 5

A bottom band two inches wide, two inches above
band of feces..... 3,500

Sept. 4 (30 days from beginning of experiment), specimens from:

Top band..... 17

Middle band 8

Bottom band 3,900

Bottom surface of pit (included earthworm casts and ant
burrows)..... 12,600

Sept. 6 (32 days from beginning of experiment), specimens from:

Surface of ground around pit, four inches wide..... 4

Region of feces..... 23,400

The duration of the experiment was thirty-two days.

The total number of larvae isolated from the bottom of the pit
and from the zone where the feces were originally deposited was
36,000 which shows that development was successful. In a zone
about two inches above the band of feces and about two inches

wide there were found 15,000 hookworm larvae, two inches higher 622 larvae. At about the middle of the wall there were found 13 larvae and at the top 17. From the surface of the ground around the pit 4 larvae were isolated. It appears from these results that the independent migration of the hookworm larvae on the wall of the pit was limited to a very narrow range. The number of larvae finally reaching the top was smaller than would have been expected from the numerous opportunities for mechanical transportation by ants and earthworms.

Application of Results to Night Soil Disposal.

The disposal of human excrement is a serious problem for all communities in warm countries. It is especially serious in rural communities where hookworm disease is prevalent. In some countries efforts to control this disease have resulted in the adoption of burial methods of night soil disposal.

In planning these experiments it was considered that thirty-six inches is the greatest depth that is likely to be generally used in the burial of human excreta. The results have shown that hookworm larvae may develop in feces buried at this depth in sandy loam soil of the type used in Porto Rico, and can migrate to the surface in large numbers.

It is very probable that under favorable conditions migration from even greater depth is possible. These results indicate that burial of feces in a loose soil is not a safe method of disposal and is of doubtful safety in some types of clay.

Burial is one of the phases in the operation of the pit latrine which is so widely used in rural communities. When the pit is nearly full of excrement the superstructure is removed and the pit is filled with earth. In view of the results of the experiments reported here it is possible that the surface over these old pits may become infested with hookworm larvae which migrated upward from the buried material. In order that it may be determined under what conditions burial of this type is safe, it is important that further studies should be conducted.

Another serious problem is the casual defecation of field laborers during the day's work. It has been customary to advise that such deposits should be covered with a few inches of earth. The results of the present experiments indicate that such a practice does not eliminate the danger of an infective spot.

One of the experiments yielded results which indicate that before the burial phase in the operation of a good pit latrine migration of hookworm larvae from the site of deposition is within a small range.

SUMMARY.

1. A series of field experiments was carried out to test the ability of buried larvae of N. americanus to migrate from various depths to the surface.
2. Larvae developed in large numbers from feces buried at depths up to thirty-six inches in sandy loam soil.
3. Larvae migrated to the surface in large numbers from material buried at various depths upto thirty six inches in sandy loam soil. Migration was equally free whether the larvae were buried after reaching maturity or feces containing ova were buried.
4. The numbers of larvae reaching the surface decreased as the depth of burial increased.
5. In the red clay, containing some sand, migration was quite successful from a depth of ten inches, but not to the same degree as in the sandy loam.
6. In a relatively pure clay migration from a depth of six inches was unsuccessful.
7. In an experiment to test the possibility of migration of larvae out of a latrine pit very few larvae were found above the level of inoculation.

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III. STUDIES ON FACTORS INVOLVED IN MIGRATION OF HOOKWORM LARVAE IN SOIL.

Introduction.

The studies on the vertical migration of hookworm larvae have thus far been concerned mainly with the fact of migration or of failure to migrate. No account has been taken of the mechanism by which locomotion is accomplished by the larvae nor have there been studies of the physical conditions which aid the progress of the larvae. The stimuli which initiate migration have been a subject of speculation but neither these nor the factors which bring about a cessation of migration have been subjected to critical experimental investigation.

It will be well to summarize here the facts bearing on these points which were brought out in the Trinidad and Porto Rico investigations. It was shown by laboratory experiments in Trinidad that in soil in which the spaces were filled with water migration did not take place. In soil with uniformly distributed moisture migration was very limited but in soil in which moisture was rising migration took place much more readily and was in the direction of the water movement. It was also shown that migration is accompanied by a rapid disappearance

of the nutritive granules from the bodies of the larvae and that the supply of these granules probably limits the range through which a larva can migrate.

In field experiments in Porto Rico migration in certain types of clay soils was unsuccessful but in a loose sandy loam with favorable temperature and moisture conditions migrations through as great a vertical distance as three feet were observed. The laboratory experiments included in this report were undertaken for the purpose of throwing light on the physical and biological questions which were raised by the previous studies.

ENVIRONMENT OF THE LARVA IN THE SOIL

For the purposes of this study it is assumed that the hookworm larvae have hatched beneath the surface of the soil or have been placed there after hatching. It has been impossible in the scope of these investigations to thoroughly study all of the factors which are concerned in the migration of the larva to the surface. The experimental evidence which has been accumulated, however, has been used as a basis for a discussion of the mechanical factors involved which is necessarily to a large extent theoretical.

A study must first be made of the environment in which the larva is now found. The soil must be considered as being made up of a very large number of solid masses, nearly all of

which are mineral grains while some are of organic matter. All of the solid particles are covered with more or less water, and the spaces between the masses are filled with soil air which contains water vapor. It is evident that the amount of air present will vary inversely with the amount of water in the soil. For the purposes of this study all of the solid particles may be classed together, since we are not yet aware of any way in which their chemical nature affects the activities of the larvae. It is with their physical properties, especially their size and shape that we are concerned. In shape and in size there are wide variations, the diameter ranging from 1 micron to 1 millimeter or more. From the fact that the diameter of a hookworm larvae is about 25 microns, it will be understood that it cannot pass through soil whose grains or their aggregates are within the low ranges. We are concerned not only with the size of the particles but with the size of the inter-spaces which separate them and with the number of contacts by which they are brought into relation with neighboring particles. These factors will vary with the arrangement of the particles or the structure of the soil. Calculations (Soyka, 1885) have been made concerning the physical characteristics of soils with certain theoretical arrangements of particles.

In these calculations the particles have been considered as spheres. In the simplest of the arrangements shown in figure I, the particles are in columns, each particle touching its neighbor at four points. It will be noted that for soil particles of a given size the interspaces are large and the points of contact relatively few. In figure II there are shown ideal soil grains in an oblique arrangement in which the size of the interspaces has been greatly reduced and the number of contacts increased. Figures III and IV show arrangements in which particles of different sizes are combined in different arrangements.

It is possible by a rather laborious process to separate the particles of a given soil sample into groups according to size. These groups have been established by arbitrary methods and the statement of the result has been termed a mechanical analysis of the soil. In making this analysis the soil is thoroughly broken up, the aggregates are separated and the whole is reduced to its constituent particles. The sorting of these particles shows us nothing in regard to their original arrangement. The arrangement of the particles is not a measurable quantity with our present knowledge, yet it is all important for a thorough understanding of many of the factors concerned in the migration of hookworm larvae through the soil. Under the circumstances we are compelled to confine ourselves to the crudest of general terms and we must leave unstudied many physical factors

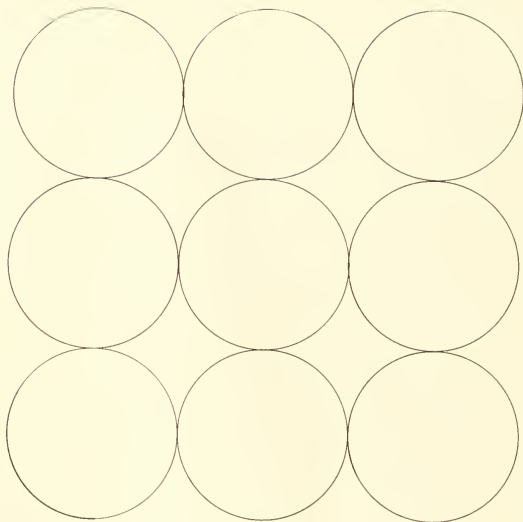


Fig. I. Possible arrangement of spherical soil particles
of uniform size.

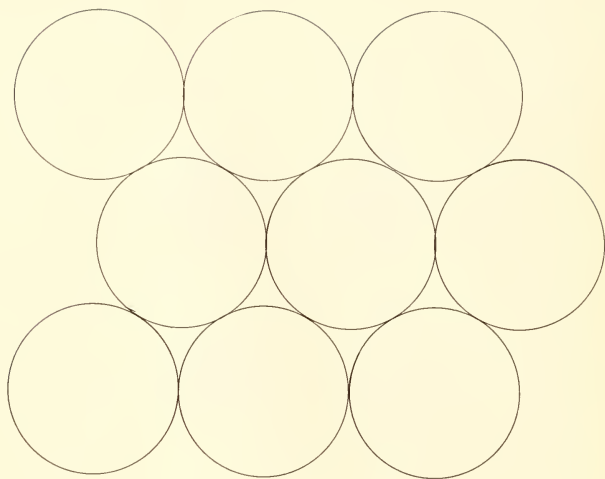


Fig. II. Possible arrangements of soil particles of uniform size.

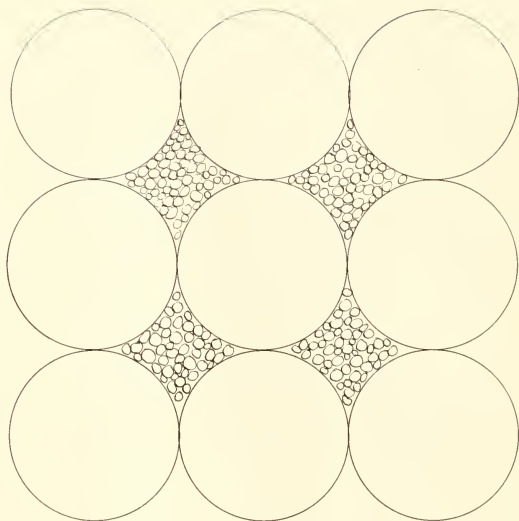


Fig. III. Possible arrangement of spherical soil particles
with interstices with soil particles.

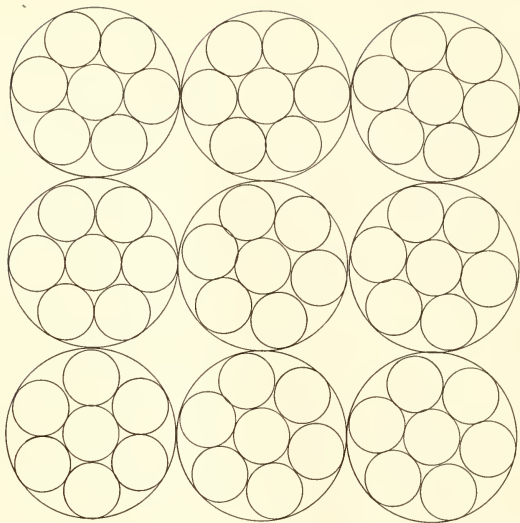


Fig. IV. Possible arrangement of spherical soil particles gathered into spherical aggregates.

about which we have little knowledge.

Valid conclusions in regard to the arrangement of soil particles cannot be drawn from the mechanical analysis because this arrangement is governed by the forces of the soil moisture. These forces are the hygroscopic, capillary and gravitational. The mechanism of the formation of the structure of the soil is given by Free (1911) as follows: "In a moist but unsaturated soil the water exists mainly in the form of thin films about the soil particles and, where the particles touch, the films are also in contact. (Briggs, 1897). At each point of contact the water films fuse, filling the annular space between the particles and forming a water mass of wedge shaped cross-section and bounded by an inwardly curved water-air surface. On this curved surface there will exist (according to well known laws) a surface tension tending to pull the sides of the wedge together, or in the case of the soil grains, to hold them together at the points of contact. It is these water-film forces which control the formation and persistence of soil aggregates and enable the preservation of more open structure than could endure in dry soil. They are the determiners of the soil structure. (Chemically cemented and other relatively permanent aggregates of soil particles are

course excluded from consideration. In their relations to physical conditions these aggregates behave like solid particles.) Without them soils would tend to assume, and in most cases would assume, the closest structure (and therefore texture) which their mechanical composition would allow."

"Furthermore, the nature and strength of these water-film forces will depend upon the water content of the soil. The absolute value of the surface tension in the wedge shaped interspaces will depend upon the curvature of the water-air surface, and it is obvious that this curvature will alter as the surface retreats toward or advances from the point of solid contact-----That is, as the water films generally decrease or increase in thickness.

The water film forces are by far the most important of the factors influencing soil structure, but there is one other which cannot be neglected. In some soils, especially those containing much clay, there are observed phenomena quite analogous to those of colloidal solutions or the so-called "true" suspension of fine particles in water.these phenomena are very complex and are still but little understood. It is probable that they are related to surface forces residing on the liquid surface between the soil particles and the soil water. They are observed only in clay soils but it is probable that they

are not confined there to." The mechanism which brings about the arrangement of the soil particles governs the size and arrangement of the spaces within the soil. It will be shown that it is upon these spaces, as well as upon the distribution of the particles, that the facility of migration depends.

Migration of hookworm larvae presupposes the presence of water in sufficient quantity to permit movement so we shall consider briefly the conditions under which the water exists, and some of the laws concerned in its movement. We have already noted that in a moist soil each particle is surrounded by a film of moisture. This film is joined to the films surrounding each of the adjacent particles. At the points of contact the masses of water are thickened into a waist-like form shown in figure V, while the surface in contact with the air is concave. The continuity of these water films extends from the water table beneath to the surface of the soil. All of this water is subject to the force of gravity but is supported by the surface tension which produces negative pressure in all of the wedge-shaped water-filled interspaces between soil particles as shown in figure V.

When the forces controlling this water are in equilibrium the negative pressure in the interspaces exactly balances the force of gravity acting upon the water, therefore the smaller the interspaces

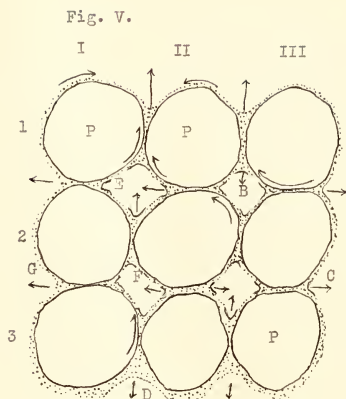


Figure V. Showing distribution of water around soil particles with the direction of forces in air-water surfaces and direction of movement of water in re-establishing equilibrium after loss by evaporation. (Modified from Lyon and Fippin. (1909) p. 171).

and consequently the greater the curvature of the surface of the wedges the greater the negative pressure and the more water supported. For this reason greater amounts and longer columns of water are supported in fine soil than in coarse soil. Changes brought about in the moisture content of one portion of the soil or in the surface tension at any point cause movements throughout the mass. Such slight changes so readily disturb the equilibrium that it may be said that the capillary moisture is never really at rest. The film increases in thickness from the surface downwards. It may be exceedingly thin at the upper limit of moisture but near the water table it becomes so thick as to very nearly obliterate the air spaces.

However thin the film may become the force of adhesion of the water to the soil particles, or the hygroscopic force, is so strong that the water is never completely removed by gravitational or capillary forces. The opposition of the hygroscopic forces to those of surface tension, gravity, and evaporation in soil which is losing water causes the progressive rupture of the bridges of moisture joining the particles until, in air dry soil, there are no moisture connections between the particles but each particle of soil has its "hygroscopic film."

The continuous movement in the water forming the capillary films to which reference has already been made is accompanied by an equally extensive movement of the air contained in the interstices

of the soil. The movements of the water and air within the soil are closely associated and in some cases stand in causal relationship one to the other. The movement of soil air may be produced by the following agencies:

1. A difference in the composition of the soil air and of atmospheric air causes an interchange of gases by diffusion.

2. Any change which may occur in the atmospheric pressure is followed by a change in the volume of the soil air, a low pressure permits the soil air to expand and escape from the soil, and increased pressure causes the atmospheric air to enter in order to equalize the pressure.

3. Changes in atmospheric temperature act in the same way as do changes in atmospheric pressure.

4. Diurnal changes in soil temperature. The soil temperature decreases from the surface downward.

5. The movement of wind, which is usually in gusts rather than of a uniform velocity, increases and decreases the atmospheric pressure and consequently there is a tendency for air to leave or enter the soil.

6. The water which enters the soil as rain must displace a volume of air equal to the rainfall which penetrates the soil and when this water is lost by the soil (by gravitation or capillary action) the same volume of air must enter.

Relation of the Hookworm Larva to the Soil Particles and
Soil Moisture.

While it has been noted by a number of observers that a hookworm larva is not necessarily killed immediately by the removal of all visible moisture, larvae in this condition are distorted and incapable of activity. In order to maintain its normal activity the larva must be surrounded by water, therefore its position in the soil must always be within the film of moisture surrounding the soil grain. That is, the larva in the soil lies on a solid substratum and is within a mass of water of variable depth which is under pressure from the interaction of various forces. Under the conditions existing in moist soil, within the zone which is likely to be occupied by the hookworm larva, the film is usually very much thinner than the larva. Therefore the larva is under direct pressure from the water-air surface of the film and is held against the substratum with whatever tension this surface is exerting on the layer of water. The pressure varies with the temperature, the chemical composition of the soil solution and the diameter of the underlying soil particle. The surface tension decreases with rise in temperature, it increases with the addition of salts to the soil solution but decreases with the addition of most of the organic oils. The soil solution usually contains both salts and organic matter and for practical purposes its surface tension may be considered to be

the same as that of water at the same temperature.

Since the pressure inside of a drop is represented by the formula $p = \frac{2T}{r}$ where p is the pressure, T is the surface tension of the liquid and r is the radius of the drop, it is obvious that with a given surface tension the pressure in the underlying water, and consequently on the hookworm larva, varies inversely with the diameter of the soil particles which it covers. As has already been mentioned, the pressure in the water which forms the waist-like bridges connecting the soil particles is negative. When the amount of water in the soil and the number of connecting bridges is so reduced that very small particles are covered with individual films the pressure on these particles may be great. Migration is apparently impossible under such conditions so we need to consider only the conditions in which relatively large aggregates, say more than a millimeter in diameter, are concerned.

The larva is not only under the influence of the forces producing pressure in the soil water but every movement in this water which is brought about by changes in the relations of these forces causes currents to impinge upon the larva. Warming of the surface of the soil lowers the surface tension and consequently releases water which is drawn downward by gravity, cooling of the surface causes the reverse condition. Evaporation at any point causes a flow of water to replace that which is lost.

Rain upon the surface or a rise in the level of the water table beneath causes changes in the forces acting which bring about the flow of relatively large quantities of water before a condition of equilibrium is re-established. The rate of flow in establishing an equilibrium depends not only on the magnitude of the differences in the forces involved but on the character of the soil. In soil with fine particles or aggregates friction is very much greater and the rate of movement of the water is consequently much slower than in coarse soils. Furthermore in such a soil the number of particles in a given cross-section is very much greater than in a coarse soil, the number of individual films is greater, and consequently the rate of movement of the water in each film is greatly reduced.

It is quite possible that the movement of soil water brings the stimuli by which the larva orients its migrations, whether they be thermal, tactile, or chemical.

The Stage in Development at which Migration Begins.

From hatching to reaching the infective stage the hookworm larva goes through very marked physiological as well as morphological changes. The activities of this period include the ingestion and storage in the body coils of nutritive material. It has been noted that during this period the type of activity of the larvae is very much different from that of the infective larvae and it is not so responsive to the stimuli which are effective in its later life.

In order to understand the physiological factors involved in migration we must know whether the larva must remain near the site of hatching on account of the food material contained in the feces or whether it can leave that site before the infective stage is reached and acquire food from the soil solution or from other sources on its travels. It is well also to know whether migration begins with the slow squirming movement of the young larva, and is initiated by stimuli to which this larva responds or whether it is not initiated until this stage is passed.

Field experiments were undertaken in Porto Rico to throw light on this question. It was found that valid conclusions could not be drawn from these experiments for two reasons: first, the buried feces attracted insects which burrowed in the cultures and so might transport larvae; second, large numbers of free living nematodes developed in and near the feces and there was great danger of confusing very young larvae of some of these animals with those of the hookworm.

A series of laboratory experiments for the same purpose was begun in Porto Rico and continued in Baltimore. The first type of experiment consisted in placing a recently recovered gravid female, Necator americanus on a slide in a drop of water and enclosing the slide in a moist chamber. The worm and its contents were examined daily. The immature larvae showed no evidence of any stimulus to leave the body of the mother. They moved very little but fed in the immediate locality of hatching. As soon as maturity was reached the

the type of activity changed. There were then much stronger movements and an evident effort of the larva to free itself from restraint. After a certain length of time it succeeded in breaking through the softened body wall.

In the second type of experiment, bits of feces which had been received in a sterile container were mixed with a little water on a microscopic slide and were allowed to stand in a moist chamber. Each of these slides was studied daily and it was noted that the immature larvae remained generally distributed over the entire slide. They did not show the well known phenomena exhibited by mature larvae of congregating at the edge of the drop.

In a third type of experiment cultures of the usual type were made with feces and previously heated soil. On top of the culture mass there was then placed a layer of previously heated soil to a depth of about five millimeters. The culture was incubated at a temperature of 27 C. Each day the surface of the overlying soil was carefully scraped, soil being removed to a depth of not more than two millimeters. The soil so removed was placed in an isolation apparatus and the culture was again covered to the original depth with previously heated soil. The sedimented material from the isolation apparatus was examined at the end of an interval of not less than four hours nor more than six hours. In no case were any larvae recovered which had not reached the infective stage. Sheathed

larvae were recovered in each case on either the fourth or fifth day.

It was concluded from these experiments that the young larva must complete its feeding and reach the infective stage before migration is begun. Therefore, studies on the basic factors of migration need concern themselves only with larvae in the infective stage.

A Study of Some of the Stimuli affecting the Activity
of *N. americanus* larvae.

Some of the crude factors which govern the motility of the infective hookworm larvae have been known from the time that the activity of these organisms began to be studied. Looss (1911, pp 425, 507 and 583) notes that motility of infective *A. duodenale* larvae is at its maximum between 30° and 38° C. He also notes that the presence of moisture is necessary for the migration which is brought about by this increased motility. In support of these observations Looss quotes experiments of his own and observations of Tenholt (1903, p. 72) in Westphalian mines and of F. Smith in Sierra Leone. The latter was probably working with *N. americanus*. Looss also emphasizes the importance of the thigmotropism manifested by the larvae. This appears to have been first described by Pintner (1905, p. 20). Difficulties of bringing about experimental conditions from which sound con-

clusions can be drawn have prevented advance in the knowledge of the precise stimuli which initiate migration of of the environmental conditions under which it is best accomplished.

In the present series of experiments it has not been possible to isolate each individual condition which might bring about a stimulus to the larvae but it has been possible to study at least three of the chief factors affecting the activity of the larvae under reasonably well controlled conditions. These factors are, the physiological age of the larvae, temperature, and contact with external objects. Under conditions more nearly approaching those in nature, and consequently less well controlled, observations were made on the effect of water currents and relation to thin films of water.

Experimental methods.

Two types of Necator americanus larvae were used for this series of experiments. One set, designated as the young larvae, had been recently isolated from cultures about one week old. They were selected for their abundant nutritive granules and they were cared for in such a manner that they would have little opportunity to use their supply of nutritive material before the experiments were carried out. The second lot, designated as old larvae, were isolated from cultures made in Porto Rico during the first week of September, 1922. Some

of these were kept in the soil of the original cultures until they were used in the experiments in November and December, 1922. Others were isolated from the cultures immediately after they reached the infective stage and were transferred to fresh soil in which they were kept until they were needed for the experiments. During this period the majority of the larvae migrated to the surface of their respective masses of soil and their activities were such that in those which survived until the time of the experiment the granules were greatly reduced in number. In a few there were only an insignificant number of granules visible with low magnification (16mm objective, x10 ocular). In others the intestinal cells contained an appreciable number of granules. In all cases the bodies of the larvae were nearly transparent and the outlines of the internal organs were clearly visible and no granules were seen except in the intestinal cells. Both the old and the young larvae were cultivated from feces obtained from different individuals.

The first stage of the study was the observation of the larva under the influence of the minimum number of external stimuli. A single larva was mounted in a Syracuse watch glass which was nearly full of water. The larva was observed with a microscope equipped with an electrical warm

stage but the current was not turned on during this part of the study. All activity was timed with a stop-watch with reference to rate and duration. For convenience the types of activity were classified as follows:

1. Quiet.
2. Restless movements of head or tail or both
but not involving entire body.
3. 1-50 oscillations per minute. It was rare that
activity involving the entire body took place
at a rate of less than 20 oscillations per
minute.
4. 50-100 oscillations per minute.
5. More than 100 oscillations per minute.

The study was begun soon after the larva was mounted but not until sufficient time had elapsed for the conditions in the surrounding medium to reach an equilibrium and for gross currents to subside. The duration of the study under these conditions varied with the activity of the larvae. Larvae which showed considerable activity were studied for an hour or more while the study was discontinued at the end of thirty minutes in cases where little or no activity was displayed. In such cases, efforts were made to start activity by means of currents produced with a pipette or by slight movements of the watch glass.

When sufficiently extended observations had been made on the

larva's activities under the conditions just described the switch connecting the warm stage was closed and the effect of the rising temperature was observed. Accurate records of the temperatures of the water were not regularly made. It is probable that the maximum reached in each case was about 36°C. The observations of activity were now repeated in precisely the same manner as before. When this stage was completed, the larva was usually set aside in the watch glass for several hours to recover from any possible effects of the activity just observed. If there had been little or no activity this precaution was not taken and the next operation was started at once.

The third part of the study consisted in the observation of the larva's activity when mounted in water on a microscopic slide under a number¹/cover glass 40X22 millimeters. The cover glass was supported with vaseline so there was some variation in the thickness of the mount and consequently in the number of contacts of the active larvae with the cover glass. Variations in the activity of the larvae which were due to lack of uniformity in thickness of the mounts were no doubt obliterated in the averages of all larvae studied. The activity of the mounted larva was first observed for a half hour or more with no current through the warm stage. The artificial light which was used as an illuminant probably produced a slightly higher temperature in this case than in the

first study in the watch glass but it is believed that this difference was not sufficient to affect the results.

Immediately after the study of the mounted larva at room temperature, the current was started through the warm stage. The study of the effect of increased temperature was begun from the time of closing the switch.

The four divisions of the study gave data to show:

1. What may be called, for the purposes of this study, the larva's basic activity. That is the activity at room temperature and with minimum stimulation.
2. The effect of an increase of temperature, other conditions remaining constant.
3. The effect of contact with external objects, slide and cover glass.
4. The effect of increased temperature associated with the same contacts previously tested.

Observations and Results.

In the course of the experiments and before any data were tabulated it became obvious that there were differences in the activity of the two lots of larvae. The differences involved types of movement, duration of activity, and response to stimuli. The young larvae usually confined themselves to a single lashing type of movement, in which the body was bent to one side to form an almost closed figure and then was bent to the other side to a

corresponding extent. The old larvae, on the other hand, varied their movements with coiling and twisting. It was quite obvious that the corresponding stimuli usually brought forth a more ready response in the young larvae than in the old and that the resulting activity was usually of a more violent character and of longer duration. These points will be brought out more definitely in the tabulated data from the experiments.

Prolonged observation of individual larvae in environments which were maintained as nearly as possible in a state of equilibrium and in which stimuli to the larvae were reduced to the minimum showed conclusively that the life of the larva is divided into at least two types of periods which may be called active and refractory. There are probably intermediate states which cannot be classified as belonging definitely to either of these. There is also a state in which the larvae is passive but is in a condition in which response can be made to a stimulus. The refractory state appears to possess considerable practical importance which will be discussed in another part of this paper so considerable attention was given to the larva when in this condition. In the earlier studies the refractory state was confused with a passive condition but it was soon recognized that any prolonged activity of the larva was likely to be followed by a condition in which the larva was not simply

quiet but could not be induced to respond to a stimulus which would have started activity in a passive larva. This condition occurred in both young and old larvae, it was independent of contact, and of the temperature conditions which were used in these experiments.

In many cases the larva in the refractory state presented no differences in appearance which would distinguish it from a passive larva. In other cases the refractory larva assumed peculiar positions, usually characterized by sharp angles^(Fig. 18 d), which sometimes were in two or more planes. These positions were maintained through considerable periods of time even though there was movement in the surrounding medium. Such a larva was often rolled across the field of the microscope without a change occurring in its contorted position. On one occasion a larva was transferred from one rereceptacle to another with no apparent change in the angles of its body. The duration of the refractory state varies widely, being sometimes less than one minute and others as long as thirty minutes. At times the larva entered the refractory state after a gradual reduction in its activity and a period of restlessness. At other times the refractory state was initiated suddenly and followed a period of marked activity without any transition. The change from the refractory to the active state was usually gradual and was characterized by awkward and jerky movements which involved portions of the body and frequently

did not change the angles which had been present during the refractory period. These movements were most suggestive of the movements of an automaton or marionette.

The stimuli which were used to test the refractory state of the larva depended principally upon contact and change of position but it was possible in some cases to bring into play the slight change of temperature which is accomplished by opening the diaphragm and illuminating the microscope with a sub-stage light. This stimulus could only be used for a larva mounted on a slide and without the warm stage. The effect of currents on the larvae was tested by means of a pipette. It was possible to do this on the slides as well as in the Syracuse watch glasses because the preparations were not sealed. Openings were left on two sides so that water might be drawn through. Shaking the watch glass or tapping the cover glass were frequently effective methods of testing the condition of the larva.

In previous experiments in which the microscope was illuminated with rather strong light from which considerable heat was evolved, it had been noted that the larvae within the field were not only stimulated to activity but that in most cases if the slide was slightly moved the larvae would move into the field again. In some cases larvae had remained in the field for ten minutes or more while the slide was

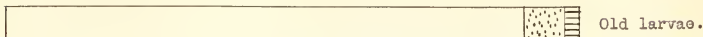
was slowly moved about in various directions. The question had arisen whether the stimulus which guided the larvae in these cases was the light or the heat. The present series of experiments have afforded observations which give valid evidence on this point. The opening in the warm stage was considerably larger than the cone of illumination. The result was that the heated zone of the Syracuse watch glass was considerably wider than the microscopic field. While there was no difficulty in keeping active larvae within the field when there was no current in the warm stage, very soon after the current was turned on the direction of the larva's travel was toward the periphery of the watch glass. This is taken to mean that the heat was a much more important factor in determining the direction of the larva than was the light. It was not determined, however, whether the stimulus itself was thermal or was a current which was produced by the change in temperature.

The study of the data obtained from the activity of individual larvae, while very interesting, would not be justified for the purposes of this paper. For this reason the results for all of the larvae of each class and for each type of environment have been taken together and from them Table I has been compiled. The same results are shown in graphic form in Figure VI. In this case the various types of activity which were observed have been represented as percentages of the total

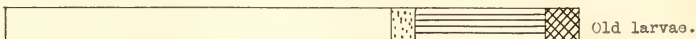
Table I. Activity of infective *N. americanus* of different physiological ages studied under varying conditions of environment.

Type of larvae	Environment	Time studied in minutes	Quiet		Restless		Type of activity 1-50		50-100 Minutes	Over 100	
			Minutes	P.C.	Minutes	P.C.	Minutes	P.C.		P.C.	Minutes P.C.
Young 11	Watch glass	899.0	416.9	46.4	23.9	3.2	15.7	1.7	410.2	45.6	28.0 3.1
Old 31	" room temp.	1718.62	1557.12	90.6	115.35	6.79	43.15	2.5	3.0	.2	0.0 0.0
Young 11	Watch glass	764.9	347.5	45.4	7.88	1.0	6.58	0.9	59.2	7.7	343.75 45.0
Old 31	" warm stage	1716.55	1156.09	67.3	73.1	4.3	390.31	22.7	97.05	5.7	0.0 0.0
Young 7	Under glass	529.23	183.7	34.7	8.68	1.6	8.5	1.6	221.95	42.0	106.4 20.1
Old 23	" room temp.	849.55	577.2	67.9	54.6	6.4	168.5	19.8	46.25	5.4	3.0 0.4
Young 7	Cover glass	416.23	131.25	31.6	2.78	0.67	1.0	0.2	6.35	1.5	273.85 66.0
Old 23	" warm stage	1113.4	465.75	41.8	23.1	2.5	425.1	38.2	127.05	11.4	67.4 6.1

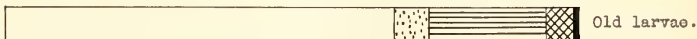
Watch glass, room temperature.



Watch glass, warm stage.



Under cover glass, room temperature.



Under cover glass, warm stage.

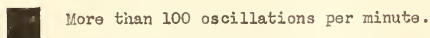
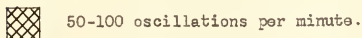
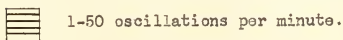
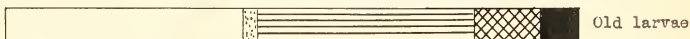


Figure VI. Graphic illustration of relative amounts of time occupied in different types of activity by infective larvae of *N. americanus* in the studies recorded in Table I.

time of observation of that particular type of larva under the given condition. It is at once obvious that the young larvae showed greater activity than the old larvae. This is shown in each environment. The activity of the young larvae in the watch glass at room temperature, the environment showing least activity, was greater than the activity of old larvae under a cover glass on a warm stage. It is also shown that a greater proportion of the activity of the young larvae is of a rapid type than is that of the old larvae. There is shown in all of the records of the old larvae more restlessness and more activity of less than fifty oscillations per minute than is shown by the young larvae. The effect of differences in environment is quite consistent in the two types. The effect of heat without external contact was in each case to stimulate the larvae to greater activity. The contact with slide and coverglass without heat produced an effect somewhat greater than did heat without contact. The combination of heat and external contact gave the most marked activity of any of the environments studied.

Other experiments were conducted under conditions which were not well controlled or where complete observations could not be made and therefore the interpretations of the results are not always clear. Following the announcement of Leiper and Khalil (1922) of the observation of the tendency of infective hookworm

larvae to travel toward a heated point on a slide, observations were made on the activity of hookworm larvae in a hanging drop. It was found that the larvae at first directed themselves unfailingly toward a hot needle held against the cover-glass a few millimeters outside the circumference of the drop. It was also noted that after a time a part if not all of the larvae failed to show this reaction. This was interpreted to be due to the preparation reaching a higher temperature so that ~~not~~ a sufficient change was ^{not} produced. In the light of further experiments it now appears that the refractory period of the larvae may account for this failure to respond.

It was demonstrated in experiments performed in Trinidad and reported previously (Payne, 1922) that upward migration was more effective in a soil in which there was an upward movement of water. This observation was confirmed in Porto Rico. In order to observe the actual migration of larvae, glass tubes filled with soil and inoculated near the bottom with infective hookworm larvae, were set in petri dishes and water was gradually added. As the water rose slowly through the soil advancing ranks of larva could be seen closely following the highest portions of the film. From time to time larvae would fail to advance as rapidly

as the film and would drop back to follow more slowly and with uncertain movements. The current of advancing water was far too weak to carry larvae of itself. This is shown by the fact that some larvae remained at the point of inoculation and later appeared in the water in the petri dish in spite of the steadily advancing water column. The interpretation of this phenomena is that those larvae which were successful were stimulated by the currents of advancing water in the films surrounding the soil particles and on the sides of the glass tubes. At the advancing edge the current in these films were most rapid and most direct and the larvae which remained in this zone received the most efficient stimuli. The larvae which dropped back found themselves in slower currents and probably in cross currents. Those larvae which for any reason were unable to go upward from the point of inoculation were caught in the thick films and water-filled spaces which formed at the base of the water column. They were deprived of their support and gradually fell to the petri dish in a manner similar to the dropping of larvae in the isolation apparatus.

Duplicate tubes 4" X 2" filled with soil were inoculated at the top with infective hookworm larvae and water was gradually added at the surface. As soon as the water reached the bottom of the tubes the material from one of the tubes was

divided into horizontal zones and the larvae were isolated from each zone. It was found that 66 percent. of the larvae recovered were in the lowest zone of one inch, 25% were in the next higher zone of 1 inch. The control tube was allowed to stand for twenty-four hours and no more water was added. Evaporation during this time was demonstrated by loss of weight. It was then divided into similar zones and the larvae were isolated. It was found that 90% of the larvae recovered were in the top zone. It is assumed that the larvae had once reached the bottom but with evaporation and the change in direction of the water column they had returned to the point of inoculation.

It has been demonstrated in this series of experiments that the statements of Looss in regard to the effect of temperature and of external contact which were based on insufficiently controlled observations were nevertheless substantially correct. The sensitivity of the larvae to external contact and to the tactile stimuli produced by currents in the surrounding media is remarkable. The evidence brought out in the experiments is sufficient to show that this type of stimulus plays a very important part in vertical migration.

It has been demonstrated that the activity of the larva is much greater at about 35°C than at room temperature. The activity at intervening temperatures has not been studied nor has it been determined what is the minimum change of temperature which will bring

about an alteration in the activity of the larva. In spite of the increased activity at higher temperatures and of the orientation of the movements of the larva toward a point of higher temperature it must be doubted that thermal stimuli as such play an important part in vertical migration of hookworm larvae in nature. In the Porto Rico field experiments larvae migrated successfully from various depths up to three feet from the surface and migration was just as successful in plots which were deeply shaded as in those which received the direct rays of the tropical sun. It is not to be supposed that at depths greater than one foot from the surface such differences of temperature will occur within the range of the sense organs of the larva as will in themselves serve to effectively orient its movements. Furthermore, successful migration was brought about in laboratory experiments in Trinidad and Porto Rico which are reported elsewhere in this paper and in previous papers. The containers which were used in these experiments varied from a half inch in diameter to four inches in diameter. It is not conceivable that there were such differences in temperature between the top and the bottom of these containers as would orient the travel of the larvae by thermal stimuli alone.

Larvae beneath the surface of the ground are constantly within the influence of currents in the capillary moisture films.

The direction of the currents may be upward, downward or lateral. Under ordinary circumstances the direction of the currents is upward during the greater part of the time, due to surface evaporation. In view of the ease with which the larva is made to respond to very light tactile stimuli it appears quite possible that the flow and direction of these currents may play a very important role in governing the migration. It is obvious, however, that our knowledge is not yet sufficient to determine what are the stimuli which initiate migration nor by what influences its direction is governed.

The assumption that the currents within the capillary films of soil water influence the direction of migration would explain the absence of active lateral migration on the surface. Currents in the capillary moisture films on the superficial particles of soil are necessarily varied and transient. There can be no such uniformity as would be necessary to produce persistent lateral travel. The same assumption would explain the observation noted in a previous paper that larvae failed to migrate vertically on the sides of an experimental pit latrine. In this case evaporation was from a vertical surface and consequently the currents of soil moisture to this surface were lateral.

THE MECHANISM OF VERTICAL MIGRATION.

In order to understand in what manner the hookworm larva accomplishes progressive movements in any position or direction the organisms were first studied on microscopic slides mounted in tap water without ^{cover}/glasses. It has been determined that in a deep drop of water when temperature conditions are practically constant and there are no external conditions which bring about movement within the drop the larva soon rests on the slide and such movements as it makes are not productive of effective locomotion. If the larva is watched for a sufficiently long time and by the intervention of some favorable condition, such as temperature changes, it finds its way to the edge of the drop, in the great majority of cases it will remain there with its head pointing outwards. If the larva is placed in a relatively shallow drop of water or if the original drop is allowed to evaporate, the type of activity of the larva changes markedly. As soon as the air-water film is at such a distance from the slide that the larva impinges upon it in its movements it is able to travel with greater speed and its movements become more purposeful. This phenomenon was noted by Looss (Looss, Vol. 2, p. 420). Looss ascribed the effective locomotion to friction between the larva and the substratum. The evidence obtained in the present study indicates that the larva obtains the necessary resistance by making use of the opposing forces of the surface film

and the solid substratum. Having once reached the edge of the drop, it is unusual for the larva to leave the zone in which the surface film curves steeply downward to meet the slide.

It has been noted that in drops of such depth that simultaneous pressure against the substratum and surface film cannot be obtained locomotion may be accomplished by pressing the head against the substratum, throwing the body forward, advancing the head and repeating the process.

At the edge of the drop it is characteristic for the larva to throw itself violently against the encircling film. This attack on the film is repeated time after time but it is only when the layer of water is very thin and consequently the resistance which can be utilized is at the maximum that it is able to cause any marked protrusion in the outline of the film (Fig. VII). There comes a time, however, in the reduction of the amount of water when the pressure apparently holds the larva or a part of the larva fast. It has been observed, for example, that a larva slipped to one end of his sheath and the empty end was caught fast by the attenuating surface film and the larva was no longer able to raise it sufficiently to re-enter. It will be understood that on a clean slide with a larva in water the last traces of water to remain will surround the larva in a very thin layer. If fresh water be brought over the larva very carefully

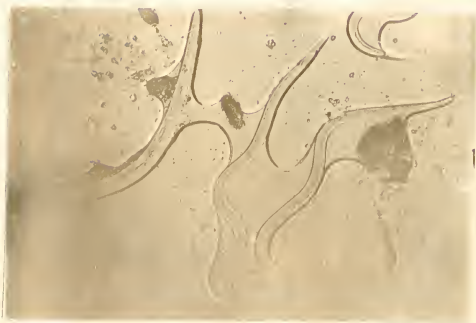


Figure VII. Larvae on slide with coverglass and small amount of water. Showing pressure of larvae against air-water surfaces and restriction of range of movements.

from the anterior end it will be noted that at first only the end of the larva is able to move while the remainder appears to be held down but becomes free as the layer of water over it becomes deeper. If there are a number of larvae near to one another on the drying slide the diminishing margin of the surface film causes the larvae to be brought closer and closer together and they are usually finally caught in a longitudinal strand and held by surface tension. (Figs. VIII).

In order to observe directly the activity of the migrating larvae, open glass tubes of various sizes from five millimeters to fifty millimeters in diameter were filled with soil. Each was inoculated near its lower end with a large number of infective larvae. The tube was then placed in a shallow vessel and water slowly added at the bottom so it rose in the soil by capillarity. The movements of the larvae were watched with the assistance of a hand lens and a binocular microscope. It was observed that the larvae moved on the soil particles in the same manner as upon the glass slide. They were enabled to move freely by the presence of an optimum amount of moisture, were caught by a marked reduction in the amount of moisture, and were apparently not able to make such effective progress in the presence of excessive moisture. Progress from one soil particle to another was usually made by the larva raising



Figure VIII. Larvae migrating from glass container in strands, supported and drawn together by surface tension of moisture of condensation. Searching position shown by tufts at upper margin.

itself perpendicular to its substratum and searching for a nearby particle (Fig. VIII^{XVII}). When contact was established the moisture films of the two particles became continuous and the larva could move from one to the other. Occasionally the larvae moved from one particle to another through the pre-existing bridges of water. It was also observed that when many larvae were present bridges from particle to particle were formed by two or more larvae. A larva moving on a soil particle would come in contact with another larva which was extended perpendicular to the surface in the searching position. The second larva would move outward along the body of the first and was able to extend somewhat beyond the head of the first so that the range of the search was extended. There were instances in which a third, a fourth and even a fifty larva took part in the search for a common footing in a new location at a higher level, the appearance of the group suggested a short rope. The larvae were closely bound together by the surface forces in the moisture surrounding them. When contact was made with the new location the moisture film at this point became continuous with that covering the larvae and they formed a living bridge between the soil particles. They were then able one after the other to take up their travels over the new path.

It was noted that when a larva crossed a gap between two particles it was apparently very difficult to raise the film/ sufficiently

to bring about continuity across the space. The first movements of the anterior portion of the body upon the new particle were deliberate but when the tail left the first particle and the continuity of the moisture was broken the body of the larva went forward to the new particle with such suddenness that it appeared to be jerked. This is probably due to the fact that in the breaking of the film surrounding the larva a new film with a very small radius of curvature was formed around the tail. The pressure at this point was greater than at any other point on the larva and the result was a forward push in which the surface film was the propelling agent.

Source of Energy for Migration.

It has been shown by experiments previously reported (Payne, 1923) that a vertical migration of at least three feet is possible for the mature larva of Necator americanus. The mere change in vertical position involves the expenditure of energy. Though it may appear that the work done in such a migration is so small as to be insignificant, if we use a very crude comparison for the sake of bringing the picture within the range of ordinary conceptions, the ascent of three feet by the larva is equivalent to a climb of about eight thousand feet by five and a half foot man.

This comparison is based on a relationship of length to distance travelled and is valueless except as a comparison of distances, yet when it is considered how inefficient are the means of the larva's locomotion and how many obstacles it must overcome it becomes obvious that its energy expenditure must be relatively^{very}/great. It has been shown in another portion of this paper that migration does not begin until maturity has been reached. At this time the larva's pharynx is closed so no more food can be ingested. Therefore the energy requirements for migration must be supplied from within the body of the larva. For this purpose there are stored very large numbers of granules which are built up during the period of feeding prior to the beginning of the second ecdysis. The nature of these granules and the locations in which they are stored have been insufficiently studied but it is generally stated of nematode larvae that the food granules are stored in the intestinal cells. In the case of Necator americanus there are evidently other points of storage because the granules in well nourished larvae extend far anterior to oesophageal bulb and so cannot be entirely in the intestinal cells. It is unfortunate that in larvae in which this condition occurs the granules are so numerous that the study of anatomical details is greatly hindered. It can now only be suggested that there

appear to be structures probably of mesodermal origin which serve for the storage of nutritive material in the mature larvae of Necator americanus. Whether there is a difference between the granules stored in the intestinal cells and those stored in other structures cannot be determined without much further study. It was suggested in an earlier paper (Payne, 1922) that the food granules in the anterior and posterior extremities of the larvae disappear before those which are located definitely within the cells of the intestine. This observation has been made in all subsequent work but the interpretation of it is still most obscure and probably depends on the determination of the nature of the granules themselves. It is quite possible that there are at least two different kinds of granules which are used differentially in supplying the requirements of the larva. It is also possible that some or all of the granules which appear to be in mesodermal structures may be stored in a part of the muscle cells themselves and if this is the case it is quite likely that such material would be used before material which it would be necessary to transport from other structures.

In order to throw some light on the nature of the granules the following experiments were carried out:

1. Larvae were stained with Sudan III by keeping them for several hours in a very dilute solution of the stain. The

The stain was freshly prepared each time by diluting a stock alcoholic solution with about ten volumes of water. This stain penetrated the unsheathed larvae and in a few cases stained the granules without undue distortion of the cells. The result showed that the well nourished larvae contained large closely packed granules which stained a dark reddish brown with Sudan III and some smaller granules which were not affected by the stain. Both types of granules were seen throughout the length of the larvae. The unstained granules were seen only near the periphery of the larvae but any which occurred in other portions would probably have been obscured by the more abundant and larger stained granules. This result indicates that a large part of the larva's nutritive store is in the form of some type of fat.

2. Larvae were placed on a slide with cover glass and a small amount of tincture of iodine was allowed to enter the preparation. Examination at intervals for several hours failed to show any different^{ial} staining of the granules. This indicates that glycogen is not stored in appreciable quantities among the nutritive granules of the larvae.

3. Dr. N. A. Cobb of the Bureau of Plant Industry, United States Department of Agriculture, kindly examined the granules in young and old larvae by means of polarized light

and demonstrated that in addition to the larger masses of fat there were present in all larvae very much finer bi-refrigrant granules. These were seen most readily in the older larvae where there was no interference from the larger granules. It appeared that the small bi-refrigrant granules were not diminished in numbers in the older larvae.

All studies of migration in this series have included careful observation of the distribution of nutritive granules within the larvae before migration and after all stages at which isolations were made. Permanent records of these observations were made in the form of photographs of the larvae studied. It was assumed that the energy which a larva is capable of expending is measured quantitatively by the abundance of the granules and therefore that the granules may be also taken as a measure of the physiological age of the larva. The number of granules being related to age in an inverse ratio. It is realized that these assumptions are subject to errors due to lack of knowledge of the precise nature of the granules but in general the observations on the number of granules present harmonized very closely with known data concerning the history and behavior of the larvae.

Larvae which have been produced from the best cultural conditions are very dark when observed by transmitted light.

The abundant food granules so completely obscure anatomical details as to render study of internal structure difficult if not impossible. The granules extend from near the tail to the most anterior portion of the larvae and are most abundant from oesophageal bulb through the middle one third of the body. At this time the granules are very coarse but there is considerable variation in size. As the larva grows older or migrates the granules disappear from the extremities and early in its life span the bulb of the oesophagus and structures anterior to it become visible. At the same time there is an increase in the transparency of the middle portion of the body. It has not been determined whether the earliest change is due to an actual decrease in the number of the granules or to a reduction in the size of the granules with consequent increase in unoccupied interstitial space. The impression is given that the latter is the case. Certainly at this stage the granules appear to be less coarse than in the earlier stage. With advancing age or with farther migration there is actual disappearance of the granules from the periphery of the middle portion of the body and a later reduction in the number and size of the granules in the intestinal cells. The granules apparently disappear from the margins of the intestinal cells first and the blocking of the individual cells becomes clear even under very

low magnification. In the terminal stages of the use of the nutritive granules they remain only in a part of the intestinal cells in the anterior portion and are visible only in lateral views of the larva. These final granules are very small and are ranged along the base of the cell on the dorsal and ventral sides of the intestine.

Early in the investigations it was noted that the disappearance of food granules, while very rapid and progressive during the course of migration, was not always proportional to the distance migrated. It was shown that under the conditions which existed in the Trinidad experiments (Payne, 1922) there was a very marked reduction in the number of food granules even after migrations of as small a distance as one inch. This observation was confirmed in Porto Rican soils with much clay present. In the more loose sandy loam of Porto Rico in which migration was so successful (Payne, 1923) such migrations gave by no means the same disappearance of food granules but even in this soil migrations of one foot or more used up a great part of the available nutritive material.

There can be little question that these variations were due to the fact that there are factors bringing demands for energy other than the net vertical distance travelled.

Among these factors are friction, energy wasted in unproductive wandering, and work done in lifting the surface film of water in searching for opportunities to pass from particle to particle.

The friction which must be overcome by the larva varies in a large measure with the pressure of the surface film and this in turn varies inversely with the size of the soil particle. Therefore, in soil with small particles the friction is greater than in soil with large particles consequently the use of food granules should be more rapid for a given distance travelled in fine soil than in coarse soil (Fig. IX). This conclusion to which one is led by theoretical considerations would cause one to anticipate that, in general, migration would be restricted as the proportion of fine particles increases. In actual experiments it was shown that in the sandy loam of Porto Rico with relatively large particles migration was rapid and reached distances as great as thirty-six inches. In Trinidad where the garden soil contained a large proportion of small particles, no migration greater than nine inches was recorded. In one of the clay soils of Porto Rico with an admixture of particles of sand migration up to ten inches was obtained but in a finer clay migration was practically inhibited. A mechanical analysis of the soils might supply additional evidence on this question. For this purpose specimens of the soils were submitted to the Bureau of Soils,

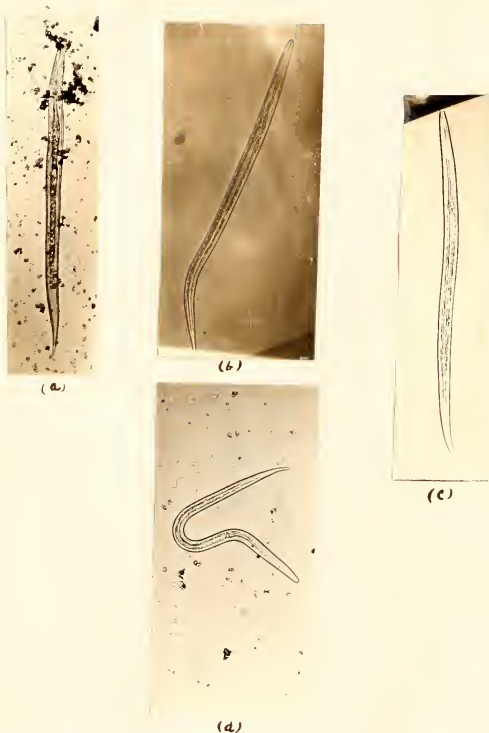


Fig. IX. Larvae which have migrated in soil of different types
(a). Trinidad garden soil-migration 6 $\frac{3}{4}$ inches.
(b) Porto Rican sandy loam, migration 8 inches.
(c) Porto Rican red clay-migration-6 inches.
(d) Porto Rican white clay. Migration 3 inches.

United States Department of Agriculture. The results have not yet been reported.

In the experiments previously mentioned in which the migration of larvae in glass tubes was watched, it was shown that under conditions of relatively stable equilibrium of the forces controlling the moisture there was much wasted energy in the attempt of the larva to migrate. Under such conditions it appeared that the larvae were not well oriented as regards direction. The larvae often wandered back and forth for a considerable length of time in a single horizontal zone before finally leaving it for a higher level. The direction of its movement might^{be} at any given instant horizontal, upwards, or downwards but the net result was nearly always upwards. It has been noted that even in cases where migration of the majority of the larvae was quite successful, a few larvae could nearly always be recovered from the lower layers of the soil and these larvae often had very few granules remaining (Fig. X). This was interpreted to mean that these larvae had failed to find the path over which they could migrate and had used up all of their available energy in wandering without making successful progress vertically.

Passage of the larva from one particle to another, either vertically or horizontally or downward, involves extending the water film from the original particle to the second particle.



Fig. X. Larvae taken from top (a) and (b) bottom layers
of Porto Rican sandy loam.

An exception to this general statement occurs in case the larva passes through one of the bridges of moisture which joins the particles (Fig. XI).

The larvae observed were obliged to lift the water--air film many times in the search for each opportunity to bridge a gap. The lifting of the film requires work, for additional area is added to the water-air surface surrounding the soil particle. The new area of surface surrounds the larva in a conical form and may be likened to a tent which the larva must support but it is applied very close to the larva except at the point where it curves outward in continuity with the surface which surrounds the soil particle. ^{Fig. XII} The extension of the surface film requires the expenditure of energy and the work done is the product of the increase in area by the surface tension. ^{the} If ~~it is~~ be calculated on the assumption that $2/3$ of the larva is extended beyond the original surface and that the increase in area is about 60% greater than the area of this portion of the larva it is found that the work done in moving the larva a vertical distance equal to its length, disregarding friction. Therefore, it appears that the reduction in food granules in migration should vary greatly according to the amount of searching of this type that is required.

The searching activity involves not only the lifting

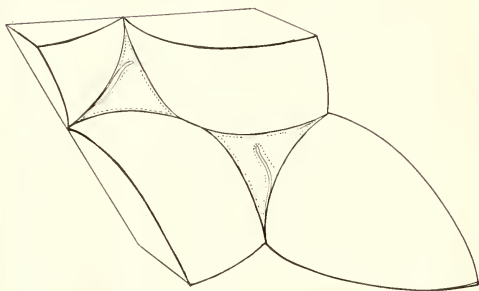


Figure XI. Schematic representation of larvae
among soil particles. (Modified from Schlichter).



Figure XII. Larva extending itself in
searching position from soil particle.

of the film but the stretching of the film during the searching movements and the support of the film during long periods of quiet in the perpendicular position. The larvae have been observed to retain this position under experimental conditions for periods of one-half hour or more and the time could probably be extended very much longer if absolutely constant conditions could be maintained. Lack of knowledge of all of the stimuli to which the larva responds prevents perfect control of such observations. It is known that on the surface the larva may remain for several weeks ready to throw itself into the erect position in response to very slight stimuli and to maintain that position for long periods of time.

The fact that the support of the water-air film requires the constant maintenance of tone in the larva's muscle and that this phenomena constitutes so large a part of the larva's activities would lead one to believe that there should be a rapid using up of nutritive material during such activity, comparable to that which occurs during migration. That this is not the case is shown by the fact that the disappearance of food granules becomes very much slower after migrating larvae have reached the surface. This indicates that there must be some means for the conservation of the nutritive material



which enables the production of the maximum amount of energy from it and therefore prolongs the larva's life. This mechanism has been sought in a study of the activity of the larva in tap water under various experimental conditions which have been described in the studies of the stimuli which influence the larva and the correlation of these observations with known facts in the physiology of other animals.

It must be obvious that if the musculature of the larva were of the striated type such as that found in man the continuous maintenance of sufficient tone to support the water-air film would require a continuous expenditure of energy at a comparatively rapid rate. Under such conditions the life of the larva on the surface, subject to many stimuli, would be a short one. Detailed histological studies of the musculature of either adults or larvae of the genus Necator are not available but it is reasonable to suppose that the cells are of the type generally found in closely related forms and described by Goldschmidt (1903 (.

This type is a modified form of the usual spindle-shaped smooth-muscle cell. We should expect that the muscles of the hookworm larva would function in the same way as the smooth muscle of some of the other animals. Investigations on the metabolism during work by smooth muscle are summarized as

follows by Bayliss (1918, page 538):

"The next question with which we are faced in the consideration of this prolonged "tonic" contraction of smooth muscle is whether the state is associated with any increase of metabolism beyond the normal one. If the muscle is held in the shortened position by a catch or ratchet mechanism, it would appear that increase is not to be expected, or of a much less degree than in tetanic contraction.

"Parnas (1910) has, in fact, made experiments which show that, in the bivalve mollusc, none is to be detected. He loaded mussels (Anodonta), whose adductor muscles had an area in section of 0.3 sq. cm. with a weight of 3,000 g. for three hours and found no increase in the respiratory exchange, either during or after the loading. Indeed, if one compares the entire respiratory ^{metabolism} exchange, of these animals, under the conditions stated, with the increase in that of a skeletal muscle of the mammal, also holding a weight of 3,000 g. per 0.3 sq. cm., it only amounts to about 0.00003 of the latter, calculated from results on the entire metabolism in man. The anodon muscle uses 0.008 mg. of oxygen per hour, as compared with some 2.8 mg. for the gastrocnemus of the cat (Verzar, 1912, p. 248):

"Take another experiment by Parnas on three specimens of Venus, which consumed 3.222 mg. of oxygen in four hours, or 0.805 mg. per hour. Loaded with 1,000 g. each for three hours,

the consumption was 0.736 mg. per hour, and, subsequently, without load for three hours, 0.311 mg. per hour. A Pecten consumed, at rest, 0.672 mg. of oxygen per hour; under a load of 500 g., 0.679 mg. per hour.

"Bethe (1911) investigated the question in another way and confirmed the view of Parnas. He found that no evidence was to be obtained of fatigue nor of loss of weight in fasting molluscs holding up a weight for a considerable time. If the consumption of carbohydrate had been comparable with that of cross-striated, skeletal, vertebrate muscle, an amount greater than the weight of the entire animal must have been burnt up.

"An interesting calculation is made by Bethe on the tonus of the arterioles in a mammal. If the mechanism were like that of the skeletal muscle, $1/6$ to $1/4$ of the whole resting metabolism of the animal would be in the arterioles.

"The strength with which a bivalve mollusc holds its shells together is known to every one who has tried to open an oyster by merely pulling the shells apart. On the face of it, there is nothing to suggest that this fact may not be due to the reflex contraction of a powerful muscle. It is found, however, that weights may be arranged to pull continuously, and yet the shells remain firmly closed against a considerable force for days. To take an example, it requires a tension to be exerted by each square centimeter of the adductor of Dioxinia exoleta equivalent to the

weight of 2,400 g. in order to close the shells against the elastic cushion which forces them open. Yet the animal can do this for twenty to thirty days continuously without evidence of fatigue (Parnas, 1910). Consideration of such facts led Grutzner (1904) to suggest that the muscle fibres cannot be exerting tensile stress by a continuous excitatory process, but that the fibres must be "hooked up" in some way, by a kind of arrangement similar to a ratchet, and kept in the position to which the shortening process brought them. If we raise a weight to a certain height and hold it suspended, we have seen that considerable work has to be done all the time, and that fatigue soon results. But if a bolt is shot out under the weight, so as to support it, it remains in the raised position without any further expenditure of energy on our part."

That such a mechanism is operative in the larvae studied as well as in the forms studied by the physiologists, who have been quoted is suggested by the refractory periods which were shown in the studies on the activity of the larvae. During these periods the larvae usually assumed positions in which they were bent in one or more planes. They usually appeared cramped or contracted. Occasionally they were straight. During such periods the larvae failed to respond to all ordinary stimuli.

When moved about by currents their position did not change or changed only very gradually. In the transition between the refractory period and the period of activity there was frequently a phase of awkward and hesitating movements as though the animal was thawing after having been frozen. The portions of the body which were sharply bent during the refractory periods were frequently the last ones to resume free activity.

By way of summary it may be said that in order to explain the slow disappearance of the nutritive granules during the activities involved in its searching movements we may assume that when the larva has performed the relatively large amount of work involved in extending the water-air surface to enable it to assume the perpendicular position its muscles become "locked" in such a manner that the position is maintained and the film supported without the use of additional energy. The waving of the larva under mild stimuli does not extend through a very large angle and apparently does not involve the entire length of the larva. Therefore, it is possible that this movement may not affect the tonus of the muscles, which are acting to support the film. The work done in waving, therefore, would be the relatively small amount involved in producing new surface on the convex side of the larva during each oscillation. The energy required for this is partly balanced by energy released through the reduction of the surface on the concave side.

The experimental work has shown that very slight stimuli cause the larva to extend outwards from their substratum or to retire. A slight addition of moisture from above or from below so alters the conditions affecting the equilibrium in the soil moisture that larvae present either retire through the influence of the stimuli or are forced down by the movements in the water. Changes in temperature are of great importance, slight warming of the surface brings about great activity and extension of the larvae from the substratum if other conditions are favorable. Warming the substratum causes larvae already extended to retire toward a lower level. Air currents bring about movements in larvae already extended, probably through evaporation and changes in the equilibrium of the forces in the soil moisture. Therefore, we may conclude that larvae which ^{are so placed as to be subject to frequent} stimulation will expend energy at a greater rate than those which are located under relatively constant conditions. The greater expenditure of energy will bring about a more rapid reduction in nutritive material and thereby shorten the life of the larvae. This assumption is experimentally confirmed by Augustine (1923) in a comparison of the length of life of larvae under conditions of frequent wetting and drying and under relatively constant conditions in deep shade. The

fact that the larvae recovered from the lower levels of migration experiments nearly always showed a great reduction in nutritive material indicates that these larvae had been active but that their activity had not resulted in effective migration. It was frequently true that larvae from the lower levels showed greater reduction in granules than those which had successfully reached the surface and had remained there. It is probable that these larvae which failed to reach the surface had encountered stimuli which brought about migration along paths which did not lead to the surface. It is possible also, that some larvae which had reached to the surface or near to it were caused to take a downward direction by temperature or moisture changes and were unable to return.

It has been observed that under a number of different conditions the larva adopts a type of activity in which the anterior end of the body, including various lengths as far back as the oesophageal bulb, was held rigid and was bent forcibly at an angle with the remainder of the body. It is assumed that this rigidity is attained through the activity of such mechanisms as the hypothetical "lock." This type of activity is adopted in the first stages of the lifting of the air-water surface, and the head is thrown out with a jerk. It is also used under certain conditions when the larva appears

to attempt to pry its way into the substratum. Although efforts to actually observe the mechanism of penetration of the skin have so far failed it appears that this may be brought about by a prying into the interstices between the superficial cells.

Summary.

1. A series of experiments was carried out to determine the relation of certain physical and biological factors to vertical migration of hookworm larvae in soil.
2. A brief account is given of the relation of soil particles to soil moisture and soil air.
3. It is shown that the larvae in the soil lie within the capillary film of moisture surrounding the soil particles. They are subject to pressure by the surface forces and to movements of moisture within the capillary zone.
4. It was shown by experiments that migration does not begin until the larva has reached the infective stage.
5. Prolonged studies of individual larvae showed:
 - a. That activity was increased by increase of temperature up to 35°C.
 - b. That activity was increased by contact with other objects.
 - c. That activity was interrupted by periods in which the larvae were

refractory to available stimuli.

- d. That young larvae showed greater activity than old under all conditions studied.

6. Evidence was obtained that the movements of the larvae were apparently readily oriented by movements in the surrounding soil water. It appeared that in the migration observed in both field and laboratory experiments this form of stimulus played a greater part in orientation than did thermal stimuli. If this is the chief orienting stimulus it goes far to explain the restricted nature of lateral migration of larvae on a horizontal surface and of vertical migration on a vertical surface, such as the wall of a pit latrine.

7. Evidence was obtained that the larva obtains resistance necessary for locomotion by making use of the opposing forces of the air-water film and the solid substratum.

8. Energy for migration must be derived from nutritive material stored as granules in endodermal and mesodermal tissues of the larvae. The nature of the granules was not determined but there appeared to be large masses of some fatty substance and smaller granules of some other material.

9. The abundance of the granules was taken as a rough measure of the physiological age of the larvae.

10. On the basis of experiments reported in this and

previous papers, certain theoretical relationships of the type of soil to the energy requirements of migration were discussed.

11. It was shown that the relatively slow utilization of nutritive material which occurred in larvae which were erect on the surface of the soil was probably due to a mechanism for the conservation of energy such as has been suggested in the activity of smooth muscle in other animals.

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IV. THE RELATION OF THE PHYSIOLOGICAL AGE OF HOOKWORM
LARVAE TO THEIR ABILITY TO INFECT THE
HUMAN HOST.

Introduction.

The demonstration of the rapid loss of food granules in infective *N. americanus* larvae in experiments conducted in Trinidad and Porto Rico at once brought up the question whether larvae in which practically all of the nutritive granules have disappeared can enter the human host and establish themselves. The importance of this problem was shown by the fact that all larvae which migrated to the surface from feces which had been buried, after the manner which has been standardized for night soil disposal in some countries, were of the type in question. It was also found in the field studies conducted at the same time and reported in this series that a large proportion of the larvae recovered from areas of soil pollution had utilized nearly all of their nutritive material. Since it is quite obvious that the larva's life span outside the host is limited by the supply of stored nutritive material, the physiological age of the larva may be roughly measured by the number of granules. For the

purposes of this study, the term young larvae was applied to those in which the granulation was abundant, obscuring the details of internal structure, while the term old larvae was applied to such larvae as had few granules. The latter were transparent and gave a clear picture of the internal organs. Looss (1911, p. 508) studied the relation of the age of larvae to the ability to penetrate the skin and his view is summarized as follows, "The age of the larvae has some influence on the realization of the skin infection in so far as old larvae seem to seize an opportunity of penetrating more eagerly than those which have only just reached the stage of maturity." He quotes Schüffner (1905, p. 689) in support of this view.

Experimental Methods.

The studies on this subject were carried out by infecting dogs with mature larvae of A. caninum and human volunteers with mature larvae of N. americanus. The major part of the experiments was carried out in Baltimore but two experimental infections were produced in volunteers while in Porto Rico as a preliminary measure. The human subjects used were adults, two of whom were free from previous infestation. The third had a light infestation

with N. americanus when the experiment was begun. Seven dogs, belonging to two litters, were used. The first litter was about six months old at the beginning of the experiment. It contained three dogs, two of which were used for experiments while the other was maintained to supply infectious material. The second litter was six weeks old when infected. It consisted of four puppies which were of about equal size and development.

Mature larvae for the experiments were obtained from cultures made in heated soil. They were isolated from the cultures on the fifth to the seventh day. Some of the larvae which were to be aged were placed in loose soil to migrate until the desired condition had been attained. Others were placed on the surface of soil and were allowed to exhaust their nutritive material when they were again isolated. Before use all larvae were washed by one or more transfers through clean water. All larvae used were carefully examined microscopically and were counted. In infecting human subjects the skin of the fore arm^{area} was used as the site of entrance, in dogs the abdominal skin adjacent to the groin was used. The microscopic slide or cover-glass on which the larvae had been counted was then inverted and placed on the chosen site. It was left in position until the fluid had evaporated, which required thirty minutes or more. Those dogs which were infected by mouth received larvae which had

been washed from the microscopic slide to pieces of bread or into a hard gelatin capsul which contained crumbs of dry bread. In order to avoid accidental loss of the larvae before reaching the stomach the first capsule was enclosed in a larger capsule.

Thorough microscopical examinations of the feces of all of the experimental subjects were made before the administration of larvae. Frequent examination of the feces was begun about two weeks after the infection. As soon as ova were found by the examination of smears the estimation of the output of hookworm eggs by the Stoll method was begun. In the majority of cases the egg counts were made by Mr. Stoll to whom I am greatly indebted for this work. In some of the subjects the entire output of feces was saved for a period of several weeks and daily egg counts were made, in other cases egg counts were made at irregular intervals. Every precaution was taken in order to approach uniformity in the experiments. The dogs were kept in separate cages under the same conditions and were fed each day the same weighed amount of dog biscuit and milk.

Experiments in Detail.

Volunteers 1 and 2. These subjects were young adults who had had no known previous infestation with any related parasite. They were used for preliminary infestations and reference has been made to them by Stoll (1923) and by Augustine (1923). In the case of Volunteer 1, twenty-five moderately nourished, unsheathed N. americanus larvae were placed on the skin of the forearm covered with filter paper, and a bandage was applied. About fifteen minutes later tingling and itching began. The bandage was retained for one hour and upon its removal twenty punctate areas of injection were counted and were interpreted as points where larvae had entered. In the case of Volunteer 2, the technique was the same and young mature larvae were used. Subjective symptoms of penetration began in about seven minutes and when the bandage was removed there were about forty lesions. Mr. Stoll made several series of egg counts on these subjects after the infestation had been established. The egg output of Volunteer 1 was about 380 eggs per gram and that of Volunteer 2, about 600 eggs per gram. The total daily egg output of eggs was about 40,000 and 100,000, the ratio of larvae applied to total daily egg output 1:1600

and 1:2000. These figures may be taken as roughly representing the egg output which is to be expected from an infestation produced by young larvae applied under the conditions which were used in these experiments to individuals who had not been previously infested.

Volunteer 3. This subject was a young adult with a light infestation represented by an egg output of about 48,000 eggs a day. On November 22, 1922, twelve transparent and inactive larvae which had been carefully selected from those remaining alive in a culture seventy-five days old were placed on the bare forearm which had been washed with soap and water. On the following morning sixty-nine similar larvae were placed on the same arm. The larvae used in this experiment were from the stock from which were selected the old larvae which were used in the activity studies previously described. There were no signs of penetration of the first larvae applied but the application of the second lot of larvae was followed in about fifteen minutes by a stinging sensation and two hours later injected points appeared adjacent to some of the hair follicles. This was

followed by a mild "ground itch" with about twenty-five lesions. This would indicate that about one-third of the larvae were able to penetrate. On the fourth day after the application of the larvae hoarseness with a slight cough developed. The laryngeal symptoms which lasted about a week, were at times so severe as to produce aphonia. The cough was paroxysmal and was severe during the first week and persisted in a milder degree for two weeks longer. Examination of the blood on November 22, showed eosinophiles 8 per cent, on November 28, 14 per cent, on March 3, 1923, 14 per cent. No counts were made during the intervening period.

Daily egg counts were begun by Mr. Stoll on this subject on December 22, 1922 and were continued through February 1, 1923. The entire fecal output was collected and weighed so that an estimate of the daily output of hookworm eggs could be made. The average daily output for each week was calculated and the results have been incorporated in Table I and they are shown graphically in Figure I. They show that there was no rise in egg production until the seventh week after the infection. The egg output rose from about 48,000 to about 86,000, an increase of 38,000. The ratio of

Table I.
egg output
 Daily average of Volunteer 3 by weeks from day
 of infection.

Date	Weeks from infection	Average daily output
First Infection		
Dec. 22-28	5	49,300
" 29-Jan. 4	6	47,400
Jan. 5 - 11	7	62,600
" 12 - 18	8	71,000
" 19 - 25	9	86,700
" 26 -Feb. 1	10	85,700
Second Infection.		
Mar. 18-24	3	89,700
" 25-31	4	84,400
Apr. 1 -7	5	117,500
" 8 -14	6	139,500
" 15 - 21	7	143,600

Figure I. Volunteer 3. Average daily egg output by weeks, following two infections with N. americanus on the skin.



of larvae applied to increase in daily egg output was 1:470.

On March 3, 1923, the same subject was infected with 81 well nourished young larvae, 27 of which were placed on the left arm and 54 on the right arm. Subjective symptoms did not appear for a half-hour and then they were less severe than in the previous infection. It was possible to distinguish about twenty lesions on the left arm and about forty on the right arm. Cough and laryngitis developed on the third day and were quite severe for one week when the onset of a mild respiratory infection masked the symptoms. The eosinophiles, which constituted 14 per cent of the leucocytes on March 3rd were 11 per cent on March 7, 12 per cent, on March 12, 14.5 per cent on March 15, 13.5 per cent on March 22, 13.0 per cent on March 29.

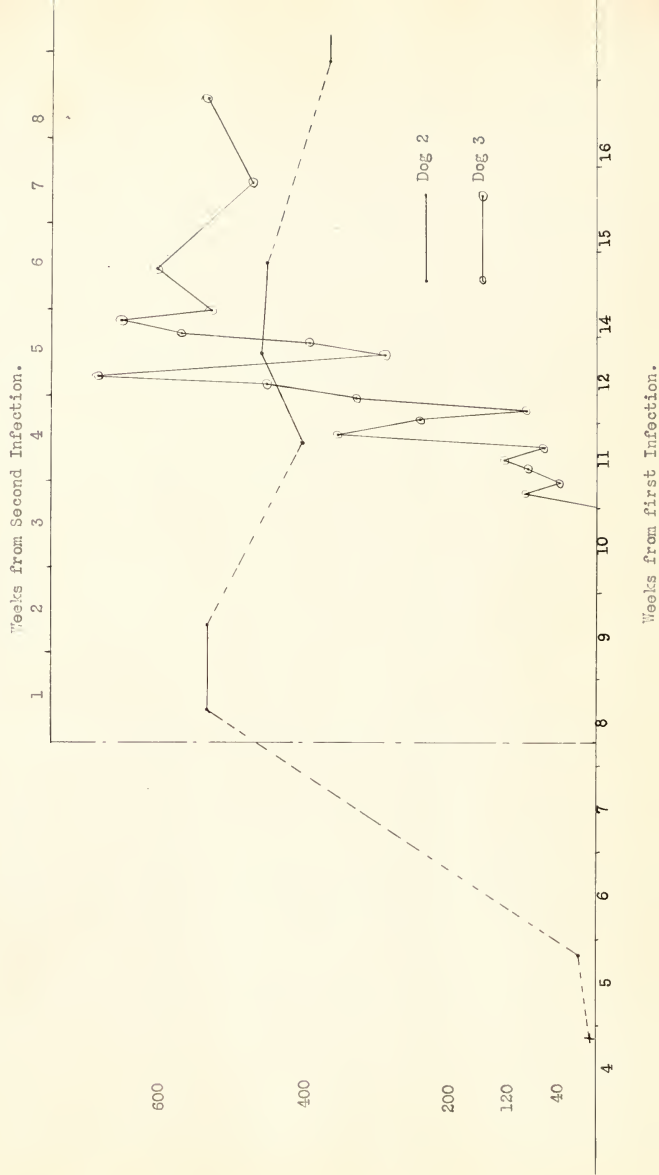
Egg counts were again begun on March 18 and the same procedure was followed as in the earlier infection. The average daily output for each week is shown in Table I and in Figure I. It is shown that the egg output had remained practically stationary from February 1st to March 18th. The rise in egg production began in the fifth week and at the end of the seventh week it had risen from 89,000 eggs per day to 143,600, an increase of 53,900 eggs

per day. The ratio of larvae applied to increase in daily output was 1:665. Although the curve of daily egg output shows a tendency to become horizontal it is possible that an additional increase may yet be shown. The daily egg counts are being continued to determine this point.

Dog 2. Age five months. November 25, 1922, 700 young larvae were applied to the skin of the groins. The dog showed no discomfort during the operation and it was not possible then or later to demonstrate lesions produced by the entrance of the larvae. One hookworm egg was found on a smear made on December 22. Egg counts were then made by Mr. Stoll at intervals during the remainder of the experiment. On December 29 the egg output was estimated to be 28,700. Estimated outputs for the remainder of the period of study are indicated graphically in Figure.II.

On January 15th the experiment was repeated, 700 young larvae being placed on the skin of the groin. A few lesions were seen. On January 29 this dog began to pass masses of blood and mucus and this was frequently repeated until the sixth week after the infection. The egg output showed no increase due to this infection but on the contrary

Figure II. Egg output of Dog 2, first and second infections. Egg output of Dog 3.
(infection simultaneous with second infection of Dog 2)



showed a tendency to diminish up to the end of the experiment. The egg output found in this dog was probably due to the first infection. It amounted to about 440,000 eggs a day. The ratio of larva applied to the resulting daily output was 1:630. On March 20 the dog was killed and there were recovered 71 A. caninum of which 37 were females and 24 were males.

Dog 3. Age six months, member of same litter as Dog 2. On January 15, ⁷⁴³~~734~~ young larvae were fed on a piece of bread. Within a half hour the dog vomited some bread and fecal material. The vomitus was placed in an isolation apparatus and 33 larvae were recovered. It was assumed that about 700 larvae were retained by the dog. January 28 nine ascarids were expelled and from this time the dog's general condition was below par, her appetite was poor and from time to time she passed masses of blood and mucus. These symptoms continued through the fifth week after the infection. A fecal smear examined on February 3rd was negative for hookworm eggs but an egg count made on February 4th by Mr. Stoll showed an output of 100,000 eggs. Daily egg counts were continued through the eighth week after infection and the data are

shown graphically on Figure 2. The daily output during the eighth week was 545,000 eggs. The ratio of larvae applied to the resulting daily egg output was 1:779. On March 10, the dog was killed and 122 A. caninum were found of which 57 were females and 65 were males.

Dog 4. Age six weeks. On February 16, 705 old larvae were placed on the skin of the groins. On the following day a few lesions were noted. No symptoms of the infestation were shown. On March 5 examination of the feces by smear and salt flotation were negative. On March 6th an egg count showed an estimated output of 2,900 eggs a day. The data shown by daily egg counts during the remainder of the experiment are to be found on Figure III. The average egg output for the last seven days of the experiment was 69,000. The ratio of larvae applied to the resulting egg output per day was 1:99. On March 26 the dog was killed and 10 A. caninum were recovered, 8 of which were females and 2 males.

Dog 5. Age six weeks same litter as Dog 4. On Feb. 16, 700 old larvae from the same lot as those which were administered to Dog 4 were fed on a piece of bread. He was watched for an hour and no vomiting occurred. On March 6th he began to pass masses of blood and mucus.

FOLD OUT

A fecal smear on March 2nd was positive and daily egg counts were begun. The data obtained is plotted in Figure 3. The average egg output of the last five days was 986,000, the ratio of larvae administered to egg output being 1:1,410. The dog was killed on March 27 and 97 *A. caninum* were recovered of which 48 were females and 49 were males.

Dog 7. Age six weeks, same litter as dogs 4 and 5. On February 19, 698 old larvae which were in a slightly better condition with respect to nutritive granules than those which were administered to dog 5 were given in a capsule. He was watched for an hour and no vomiting occurred. Diarrhoea with blood and mucus began on March 7 and continued at intervals throughout the experiment. His appetite was often very poor. The first positive fecal smear was obtained on March 5th, daily egg counts were begun and the results are shown in Figure III. The average egg output of the last four days of the experiment was 807,500, the ratio of larvae administered to the resulting egg output being 1:1,157. On March 29 the dog was killed and 168 *A. caninum* were found, of which 89 were females and 79 males. In this

case the worms were distributed throughout the length of the small intestine while in the previous cases they were more concentrated in the upper half of the organ.

Dog 6. Age six weeks, same litter as dogs 4, 5 and 7. On February 19, 700 young larvae were administered in a capsule. He was watched for one hour and no vomiting occurred. This dog's appetite was poor throughout the experiment, blood and mucus was noticed at the beginning of the second week and continued for the remainder of the experiment. A fecal specimen was found positive on March 5, and daily egg counts were begun. The results are given in Figure III. The average egg output of the last six days of the experiment was 2,238,000, the ratio of larvae administered to the resulting egg output being 1:3,197. On March 28 the dog was killed and 379 A. caninum were recovered, of which 191 were females and 188 were males. The worms were distributed throughout the small intestine and a few were found in the large intestine.

Discussion of Results.

In this series of eleven experimental infections, four of which were with N. americanus and seven with A. caninum there was only one in which there was an apparent failure of the larvae to establish themselves. This was in one of the older

dogs which had an active infestation of seven weeks duration at the time of the unsuccessful inoculation. The larvae which failed were young and were apparently in excellent condition so the reason for the failure must be sought in some factor other than physiological age.

In the Volunteers who were infected with N. americanus it was unfortunately impracticable to obtain the worm index because it was necessary to use these experimental infestations for other purposes. The comparisons used in this study must be based on the ratio of the larvae applied to the skin to the resulting egg output. The data in Table II show that in the cases Volunteer 1 and Volunteer 2 who received as their first infection young larvae this ratio was 1:1,600 and 1:2,000. In Volunteer 3 an infection with old larvae gave a ratio of 1:470 while a subsequent infection with young larvae gave a ratio of 1:665. It is calculated by Stoll that 17 worms or 68 per cent of the larvae applied reached maturity in Volunteer 1. The estimated number of worms in Volunteer 2 is 28 or 56 per cent of the larvae applied. Applying the same factors to the data from Volunteer 3, who had a previous infestation it appears that from the infection with 81 old larvae 9 worms or 11 per cent

Table II.

The summary of data obtained in infection experiments
with young and old larvae of N. americanus
and A. caninum.

Type of larvae	No. used	Subject	How inoculated	Appearance of ova	Ratio of No. larvae to daily egg output	No. of worms found
<u>N. americanus</u>						
Young	25	Vol. 1	skin	-----	1:1600	-----
"	50	" 2	"	-----	1:2000	-----
Old	81	" 3	"	7th week	1: 470	-----
Young	81	" 3	"	5th "	1: 665	-----
<u>A. caninum</u>						
Young	700	Dog 2	skin	27 days	1: 630	71
"	700	" "	"	No increase	----	-----
"	700	" 3	mouth	20 days	1: 779	122
Old	705	" 4	skin	17 "	1: 99	10
"	700	" 5	mouth	14 "	1:1410	97
"	698	" 7	"	14 "	1:1157	168
Young	700	" 6	"	14 "	1:3197	379

came to maturity, while from the subsequent infection 14 worms or 17 per cent had come to maturity at the end of the seventh week. The disparity between the percentages coming to maturity in the first two cases and in the third indicate that there was some factor other than physiological age which was operative but the difference in the percentage of the two types of larvae which established themselves in Volunteer 3 demonstrates a greater power to infect in the young larvae than in the old. This is borne out in the experiments with A. caninum in dogs. This conclusion is also in harmony with the results of studies[§] on the differences in the ^{activity} in young and old larvae. When it is considered that the old larvae which were used in these experiments were both physiologically and actually the oldest larvae which it has been possible to obtain for the purpose and were as old as are likely to be encountered in numbers in the field, it must be recognized that some power to infect is retained to the end of the larva's life span.

The data which ~~was~~^{were} obtained in the experiments with A. caninum may be simply tabulated as follows:

Age of larvae	Subject	Age	How inoculated	No. of worms found	P. C. of larvae established
Young	D 2	5 mos.	skin	71	10
"	D 3	6 "	mouth	122	18
"	D 6	6 wks.	"	379	54
Old	D 4	"	skin	10	1
"	D 5	"	mouth	97	14
"	D 7	"	"	168	24

Considering first the young dogs, which were all of the same litter, and were believed to have had no previous infestation with hook-worms, the greater number of young larvae which came to maturity was striking. It is also obvious that where the same larvae were used by the same route of infection but in dogs of different ages as in dog 3 and dog 6, in which young larvae were used by mouth the greater infestation was obtained in the younger dog. This bears out statements of Looss (1911, p. 506). Where dogs of the same age received similar larvae by different routes the greater infestation occurred in the dog receiving larvae by mouth.

These experiments add some evidence to the work of previous observers on the time required for ova to appear after skin and mouth infections. The time required for the development

of N. americanus in an infection with old larvae was seven weeks while in a subsequent infection in the same subject with young larvae it was five weeks. The time required by A. caninum varied from 14 to 27 days. There was no significant variation apparent between young and old larvae in the younger dogs. The development of both types of larvae was more rapid in the young dogs than in the older ones.

It has been shown in the case of Volunteer 3 with N. americanus and Dog 2 with A. caninum that in the presence of a prior infestation the development of larvae was not so great as in other subjects in which such prior infestation was not present. Many more cases would be required to establish this observation as a constantly occurring phenomenon. Neither can anything more than suggestion be made as to the mechanism by which this failure to develop may have occurred. It cannot be said whether the larvae themselves met with additional resistance during the time spent within the tissues of the host, whether the environment of the infested intestine was unsuitable, or whether the arrival of a new lot of larvae produced an intestinal disturbance which resulted in the

expulsion of some of the worms which were already established. In a number of the dogs it was noted that there were intestinal disturbances at the time when worms were beginning to come to maturity. At this time there was evidence of tenesmus and the expulsion of considerable quantities of blood and mucus. In three cases ascarids were expelled during such a disturbance. It appears to be of considerable practical importance that a study should be conducted to determine whether the existence of a hookworm infestation renders the establishment of additional worms more difficult. The demonstration of such a phenomenon would throw light on the disparity which is known to exist between the exposure of laborers to infection in some countries and the known rate of acquiring additional worms to which attention has been called by Darling (1922).

Summary.

1. Experimental infections were produced in three human subjects with N. americanus larvae and in six dogs with A. caninum larvae in order to test the ability of larvae which had utilized nearly all of their nutritive material to infect the host as compared with

the ability of young and well nourished larvae.

2. Larvae were artificially aged by migrations under laboratory conditions.

3. The experiments with N. americanus indicate that even the oldest larvae obtainable retained some power to infect but this power was not so great as that possessed by the young larvae.

4. The experiments with A. caninum confirmed the results of the experiments with N. americanus.

5. Evidence was obtained that a prior infestation may have some influence in rendering the establishment of a new infestation more difficult.

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